UNIVERSITÁ DEGLI STUDI DI MILANO-BICOCCA
Facoltà di Scienze Matematiche Fisiche e Naturali
Dipartimento di Biotecnologie e Bioscienze
Dottorato in Biotecnologie industriali, XXVI ciclo

Role and regulation of homologous recombination in response to DNA double strand breaks and replication stress in *Saccharomyces cerevisiae*

Coordinatore: Prof. Marco Ercole Vanoni
Tutor: Prof.ssa Maria Pia Longhese

Falcettoni Marco Cesare
Matr. 074219

Anno Accademico 2012-2013

SUMMARY

RIASSUNTO

La ricombinazione omologa, HR dall'inglese Homologous Recombination, è un meccanismo fondamentale per garantire il mantenimento della stabilità genomica in quanto è richiesta per riparare diversi tipi di lesione al DNA e per recuperare le forche stallate o collassate. Tuttavia la ricombinazione omologa è potenzialmente dannosa in quanto capace di generare intermedi non riparabili o riarrangiamenti cromosomici che possono portare all'instabilità genetica e al cancro. È perciò fondamentale che la ricombinazione omologa sia finemente regolata a seconda del tipo cellulare, della fase del ciclo cellulare, della regione cromosomiale e del tipo di stress a cui il DNA è sottoposto.

Tra le lesioni al DNA che diventano substrato della ricombinazione omologa vi sono le rotture a doppio filamento o Duoble-Strand Breaks (DSBs). In alternativa alla ricombinazione omologa, le rotture a doppio filamento di DNA possono essere riparate da un secondo meccanismo, il Non-Homologous End Joining (NHEJ). Mentre col NHEJ le due estremità della rottura vengono rilegate direttamente, la HR utilizza una sequenza di DNA omologa come stampo per la riparazione generando prodotti di ricombinazione che si distinguono in crossover o noncrossover in base alle sequenze parentali fiancheggianti il sito di danno. Inoltre, un DSB posizionato tra due sequenze ripetute dirette può essere riparato tramite un particolare meccanismo di ricombinazione omologa chiamato *single-strand annealing* o SSA, che risulta nella riparazione della lesione con concomitante perdita di una delle due ripetizioni e della regione compresa tra esse. Tutti i meccanismi di HR iniziano con un esteso processamento in direzione 5'-3' (noto come resection 5'-3') delle estremità della lesione per generare code di DNA a singolo filamento in 3' (*single-stranded* DNA o ssDNA), che sono legate dalla proteina di replicazione A (*Replication Protein A* o RPA). Successivamente, RPA viene spiazzata da Rad51 a formare filamenti nucleo proteici che possono catalizzare l'appaiamento e l'invasione di una doppia elica di DNA

omologa. Ogni ciclo cellulare è formato da una serie di eventi la cui successione è regolata dall'attività di una chinasi ciclina dipendete, Cdk1 in *Saccharomyces cerevisiae,* che oscilla nel corso del ciclo. Cdk1 regola anche la scelta tra NHEJ e HR durante il ciclo cellulare. La HR è generalmente limitata alle fasi S/G2, quando il DNA è stato replicato e il cromatidio fratello è disponibile come stampo per la riparazione. Cdk1 è più attiva in queste fasi del ciclo e promuove la HR stimolando la degradazione nucleolitica in direzione 5'-3' delle estremità del DSB. Non era noto se Cdk1 regolasse anche altri passaggi richiesti per la HR. Per rispondere a questa domanda abbiamo indagato quale fosse la richiesta di Cdk1 nell'esecuzione di diversi processi di HR in *S. cerevisiae*. Per aggirare la richiesta di Cdk1 per la resection, abbiamo utilizzato cellule prive dell'eterodimero Yku e/o della proteina di checkpoint Rad9, che sono entrambi noti come regolatori negativi della resection al DSB. Abbiamo dimostrato che cellule *yku70Δ*, che accumulano ssDNA alle estremità di un DSB indipendentemente dall'attività di Cdk1, sono in grado di riparare un DSB per SSA in fase G1, quando l'attività di Cdk1 è bassa. Questa capacità di riparare per SSA dipende dal processamento delle estremità del DSB, poiché tanto l'efficienza di resection quanto quella di SSA aumentano in cellule *yku70Δ* in G1 prive anche di Rad9. Abbiamo inoltre osservato che in cellule *yku70Δ* e *yku70Δ rad9Δ* in G1 si generano prodotti noncrossover come risultato della ricombinazione intercromosomica, indicando che la generazione di ssDNA al DSB è sufficiente per compensare la richiesta di Cdk1 per portare a termine anche questo tipo di ricombinazione. Al contrario, cellule *yku70Δ* e *yku70Δ rad9Δ* sono specificamente difettive nella formazione di prodotti crossover come risultato della ricombinazione intercromosomica quando l'attività di Cdk1 è bassa. Pertanto, Cdk1 promuove la riparazione di DSB per SSA e ricombinazione noncrossover agendo principalmente a livello della resection, mentre ulteriori passaggi richiedono l'attività di Cdk1 per generare prodotti crossover. Dato che

durante il ciclo cellulare mitotico i crossover possono potenzialmente portare a pericolosi riarrangiamenti cromosomici quando il cromatide fratello non viene usato come stampo per la riparazione, questa richiesta addizionale di Cdk1 nel promuovere la formazione di prodotti crossover può fornire un ulteriore meccanismo di sicurezza per garantire la stabilità genomica.

Le cellule sono particolarmente esposte ai danni al DNA durante la replicazione in quanto ogni lesione al DNA è potenzialmente in grado di bloccare la forca causando uno stress replicativo. Anche durante la sintesi del DNA la ricombinazione omologa ha un ruolo fondamentale per garantire il mantenimento della stabilità genomica in presenza di uno stress replicativo. La HR funziona in sinergia con il checkpoint di fase S e la progressione del replisoma col fine di garantire la stabilità delle forche replicative e di conseguenza una sintesi del DNA fedele.

Il complesso etretrimerico evolutivamente conservato MRX (Mre11–Rad50–Xrs2) di lievito, MRN (Mre11–Rad50–Nbs1) in mammifero, è coinvolto nella ricombinazione omologa e nel mantenimento della stabilità del replisoma. Una volta legato al DNA il complesso MRX è in grado di svolgere sia funzioni strutturali che enzimatiche, grazie alle quali partecipa a diversi processi del metabolismo del DNA. È stato dimostrato che il complesso MRX è importante durante la sintesi del DNA in quanto è essenziale nei vertebrati e la sua mancanza causa sensibilità agli stress replicativi in lievito. In particolare la delezione di qualsiasi sua subunità causa sensibilità all'idrossiurea (HU), un inibitore della ribonucleotide reduttasi che porta all'abbassamento dei livelli di nucleotidi e al blocco della replicazione. Per meglio comprendere perché la distruzione del complesso MRX causa sensibilità agli stress replicativi abbiamo messo a punto uno screening genetico per cercare soppressori extragenici dell'idrossiurea sensibilità di cellule *mre11Δ*. Abbiamo trovato due soppressori indipendenti portanti mutazioni recessive nel gene *TRR1. TRR1* codifica per la tioredossina reduttasi

citoplasmatica, un enzima noto per funzionare assieme alle tioredossine (Trx1 e Trx2 in *S. cerevisiae*) in un sistema molto conservato nel corso dell'evoluzione e noto per regolare la ribonucleotide reduttasi e la risposta agli stress ossidativi.

Abbiamo chiamato i due alleli mutati del gene *TRR1, trr1-2 e trr1-6.* Ognuno porta una singola sostituzione nucleotidica responsabile delle mutazioni puntiformi A18D e I116S negli alleli *trr1-2 e trr1-6* rispettivamente. Entrambe queste mutazioni riguardano due residui molto conservati evolutivamente e cadono nel dominio di legame al FAD. Abbiamo dimostrato che queste mutazioni causano una pedita di funzione della tioredossina reduttasi. Dato che uno dei ruoli principali del sistema delle tioredossine è la regolazione dell'attività della ribonucleotide reduttasi abbiamo provato a variare i livelli di deossiribonucleotidi (dNTP) per capire se la soppressione dell'idrossiurea sensibilità dei mutanti *mre11Δ* potesse essere legata alle oscillazioni dei dNTPs nel corso del ciclo cellulare. Abbiamo dimostrato che né l'aumento né la diminuzione nei livelli dei nucleotidi erano responsabili della soppressione. Dato che la maggior parte delle funzioni di Trr1 note passano attraverso le tioredossine abbiamo studiato se Trx1 e Trx2 erano coinvolte nella soppressione dell'idrossiurea sensibilità di cellule *mre11Δ* osservando che nei nostri mutanti di Trr1 i livelli di entrambe Trx1 e Trx2 aumentavano se comparati con quelli di cellule selvatiche. Tuttavia, nonostante queste variazioni le tioredossine non erano coinvolte nella soppressione dell'idrossiurea sensibilità mediata dalle mutazioni in *TRR1*. Questo risultato ci permette di ipotizzare una nuova funzione di Trr1 non ancora nota. Abbiamo quindi scoperto che la soppressione dell'HU sensibilità mediata dalla tioredossina reduttasi non è specifica per *mre11Δ* ma anche per diversi mutanti difettivi nei processi di ricombinazione omologa (*rad51Δ, rad52Δ* e *sae2Δ*). Al contrario mutazioni in *TRR1* non sono in grado di sopprimere l'idrossiurea sensibilità di mutanti di checkpoint (*mec1Δ* e *mrc1Δ*). Perciò l'attività di Trr1 è dannosa durante

uno stress replicativo in cellule difettive nei processi di ricombinazione. Sebbene l'idrossiurea sensibilità dei mutanti di checkpoint non è soppressa da mutazioni in *TRR1* abbiamo dimostrato che il checkpoint non è richiesto per la soppressione all'idrossiurea dei mutanti di ricombinazione. È noto che cellule difettive nei processi di ricombinazione accumulano lesioni al DNA durante uno stress replicativo. Abbiamo dimostrato che la perdita di funzione di Trr1 previene sia la formazione che l'accumulo di rotture a doppio filamento in mutanti di ricombinazione trattati con idrossiurea. Abbiamo infine osservato che, a differenza di cellule selvatiche, cellule difettive nei meccanismi ricombinativi rilasciate da un trattamento con alte dosi di HU erano incapaci d competarei la divisione nucleare mentre mutazioni in *TRR1* aumentavano la frequenza degli eventi di divisione nucleare dei mutanti di ricombinazione. Assieme questi risultati dimostrano che l'attività della tioredossina reduttasi è dannosa durante uno stress replicativo in cellule difettive nella ricombinazione omologa, al contrario, difetti nell'attività di Trr1 migliorano la divisione nucleare di mutanti di ricombinazione trattati con HU. Sebbene il meccanismo molecolare che lega la tioredossina reduttasi con ricombinazione omologa rimane da chiarire ipotizziamo che la perdita di funzione di funzione di Trr1 potrebbe promuovere dei meccanismi riparativi alternativi alla ricombinazione oppure prevenire la formazione di strutture substrato della ricombinazione.

ABSTRACT

Homologous recombination (HR) is a key pathway to maintain genomic integrity from one generation to another (meiosis) and during ontogenic development in a single organism (DNA repair). Recombination is required for the repair or tolerance of DNA damage and the recovery of stalled or broken replication forks. However, recombination is also potentially dangerous as it can lead to gross chromosomal rearrangements and potentially lethal intermediates. For this reason, recombinational events must be strictly regulated depending on the organism, cell type, cell-cycle stage, chromosomal region, as well as the type and level of genotoxic stress.

HR participates in repair of DNA double-strand breaks (DSBs), which are among the most dangerous kinds of DNA lesions. Alternatively to HR, DSBs can be repaired also by non-homologous end joining (NHEJ). While NHEJ directly relegates the broken DNA ends, HR uses homologous DNA sequences as a template to form recombinants that are either crossover or noncrossover with regard to flanking parental sequences. Furthermore, a DSB flanked by direct DNA repeats can be repaired by a particular HR pathway called single-strand annealing (SSA), which results in DSB repair with concomitant deletion of one repeat and of the intervening sequence. All HR processes initiate with extensive 5’ to 3’ end-processing (a process referred to as 5’-3’ resection) of the broken ends to yield 3’-ended single-stranded DNA (ssDNA) tails, which are bound by Replication Protein A (RPA). RPA is then displaced by Rad51 to form nucleoprotein filaments that can catalyse homologous pairing and strand invasion.

The choice between NHEJ and HR pathways is tightly regulated during the cell cycle. Eukaryotic cell division cycle comprises a series of events, whose ordering and correct progression depends on the oscillating activity of cyclin-dependent kinases (Cdks), which safeguard timely duplication and segregation of the genome. It has been demonstrated that Cdks promotes DNA repair by homologous recombination (HR) restricting this process to S/G2 cell cycle phases, when DNA has been replicated and a

sister chromatid is available as a repair template. It has been demonstrated that Cdks promotes DNA repair by homologous recombination (HR). Cdk (Cdk1 in *Saccharomyces cerevisiae*) activity initiates HR by promoting 5'–3' nucleolytic degradation of the DSB ends. Whether Cdk1 regulates other HR steps was unknown. To address this question, we explored the Cdk1 requirement in the execution of different HR processes in *S. cerevisiae*. In order to bypass the Cdk1 requirement for resection we analyzed cells lacking Yku heterodimer and/or the checkpoint protein Rad9, which are known as negative regulators of DSB resection. We showed that *yku70Δ* cells, which accumulate ssDNA at the DSB ends independently of Cdk1 activity, are able to repair a DSB by SSA in the G1 cell cycle phase, when Cdk1 activity is low. This ability to perform SSA depends on DSB resection, because both resection and SSA are enhanced by the lack of Rad9 in *yku70Δ* G1 cells. Furthermore, we found that interchromosomal noncrossover recombinants are generated in *yku70Δ* and *yku70Δ rad9Δ* G1 cells, indicating that DSB resection bypasses Cdk1 requirement also for carrying out these recombination events. By contrast, *yku70Δ* and *yku70Δ rad9Δ* cells are specifically defective in interchromosomal crossover recombination when Cdk1 activity is low. Thus, Cdk1 promotes DSB repair by SSA and noncrossover recombination by acting mostly at the resection level, whereas additional events require Cdk1-dependent regulation in order to generate crossover outcomes. As crossovers during mitotic cell growth have the potential for deleterious genome rearrangements when the sister chromatid is not used as repair template, this additional function of Cdk1 in promoting crossovers can provide another safety mechanism to ensure genome stability.

Cells are particularly vulnerable to DNA damage during DNA replication because virtually all forms of DNA damage block DNA replication causing replication stress. Homologous recombination has pivotal roles in maintenance of genome integrity also

during replication stress. HR machinery must be coordinated with S-phase checkpoint and replisome progression in order to ensure its stability during DNA synthesis.

Among factors involved in both HR and replisome stability there is the conserved eterotrimeric complex MRX (Mre11–Rad50–Xrs2) in yeast, MRN (Mre11–Rad50–Nbs1) in mammals. This complex has different roles in DNA metabolism, as it is able to bind DNA and exerts architectural and catalytic functions. It has been demonstrated that this complex is important during DNA synthesis since it is essential in vertebrate and its disruption causes sensitivity to the replication inhibitor hydroxyurea (HU) in yeast. To better understand why MRX deficient cells are sensitive to replication stress we performed a genetic screening searching for spontaneous extragenic mutations that suppress the HU sensitivity of *mre11Δ* cells. We discovered that recessive mutation in *TRR1* gene was able to partially suppress the HU sensitivity of *mre11Δ* strain. *TRR1* encode for cytoplasmic thioredoxin reductase (Trr1), a key regulatory enzyme which cooperate with thioredoxins (Trx1 and Trx2) to forming a system involved in regulation of ribonucleotide reductase enzyme and in cellular response against oxidative.

We found two mutated alleles of *TRR1* (*trr1-2* and *trr1-6*) each of one with a single base pair substitution which caused the amino acid substitutions A18D and I116S in *trr1-2* and *trr1-6* respectively. Both this mutation are in the Trr1 FAD binding domain and involve residues that are highly conserved in thioredoxin reductases from different organisms. We demonstrated that these mutated alleles encode for loss of function variants of thioredoxin reductase enzyme.

Since thioredoxin system has a fundamental role in regulation of ribonucleotide reductase enzyme we tried to modulate the amounts of deoxyribonucleotide (dNTP) in order to understand if HU sensitivity suppression of *mre11Δ* cells could be linked to dNTPs levels during cell cycle progression. We showed that nor the increase neither

the reduction of dNTPs amounts were responsible for the *mre11Δ* HU sensitivity suppression. As most of Trr1 functions required thioredoxins activities we tested if *S. cerevisiae* thioredoxins (Trx1 and Trx2) were involved in the *mre11Δ* cells HU sensitivity suppression. We demonstrated that loss of function mutations in *TRR1* gene allele caused an increase in the amounts of both Trx1 and Trx2. However, we showed that variation in thioredoxins levels were not responsible for suppression of *mre11Δ* HU sensitivity. Then, we found that *trr1* mediated HU sensitivity suppression was not specific for *mre11Δ* cells but was able to suppress the HU sensitivity of several mutants defective in HR pathway (*rad51Δ*, *rad52Δ* and *sae2Δ*) but not that of checkpoint mutants (*mec1Δ* and *mrc1Δ*). This demonstrated that Trr1 activity is deleterious during replication stress in the absence of functional recombination machinery. Although HU sensitivity of checkpoint mutants was not suppressed by loss of Trr1 function we showed that S-phase checkpoint was not involved in *trr1* mediated HU sensitivity suppression of recombination mutants. In order to test if Trr1 activities were dangerous in cells defective in HR machinery which undergoes to replication stress we demonstrated that loss of functions of Trr1 partially prevented both the formation and accumulation of DSB lesions in HR mutants during HU treatment. Finally, we showed that loss of functions of Trr1 increased the frequency of nuclear division events of recombination mutants released from HU treatment. Thus, we concluded that thioredoxin reductase activities are dangerous during replication stress in the absence of recombination. We proposed that thioredoxin reductase inactivation could promote some HR alternative mechanisms or prevents the accumulation of HR substrates improving the nuclear division of recombination mutants which undergoes to replication stress.

INTRODUCTION

HOMOLOGOUS RECOMBINATION

Homologous recombination (HR) is a key pathway to maintain genomic integrity between generations (meiosis) and during ontogenic development in a single organism (DNA repair). During meiosis, HR is essential to establish a physical connection between homologous chromosomes, thus ensuring their correct disjunction at the first meiotic division. In addition, meiotic recombination promotes genetic diversity by creating new combinations of maternal and paternal alleles. In mitotic cells the primary function of HR is to maintain genome integrity. Indeed, HR is required for the repair or tolerance of exogenous DNA damage and for the recovery of stalled or broken replication forks (Li and Heyer, 2008). Not surprisingly, defects in HR and associated processes define a number of human cancer predisposition syndromes associated with genome instability. However, recombination is also potentially dangerous as it can lead to gross chromosomal rearrangements and potentially lethal intermediates (Kolodner et al., 2002). The balance between too little and too much recombination must be strictly regulated depending on the organism, cell type, cell-cycle stage, chromosomal region, as well as the type and level of genotoxic stress (Heyer et al., 2010).

Homologous recombination mechanism: HR is promoted by the formation of a DNA double strand break (DSB), the most deleterious DNA lesion, whose failure
to be repaired can lead to loss of genetic information and chromosome rearrangements. DSBs can arise accidentally during both mitosis and meiosis of eukaryotic cells, either by DNA replication problems or by exposure to environmental factors, such as ionizing radiations or genotoxic drugs. Moreover, they are introduced

into the genome in a programmed manner to initiate meiotic recombination in germ cells.

When a DSB is induced in a cell, the DNA ends are resected in a process that involves several nucleases and that has been dissected in *Saccharomyces cerevisiae*. End resection is initiated by the conserved complex called MRX (Mre11-Rad50-Xrs2) in yeast and MRN (Mre11-Rad50-Nbs1) in mammals, which together with Sae2/CtIP can remove oligonucleotides from the 5' strand, resulting in limited end processing (Mimitou and Symington, 2008; Zhu et al., 2008). MRX complex is required to recruit Dna2, Exo1, and Sgs1 to the break site (Shibata et al., 2011). More extensive resection is carried out by the 5'-3' exonuclease, Exo1, or by the combined activities of the Sgs1-Top3-Rmi1 complex (STR) and Dna2 (Mimitou and Symington, 2009). Once resection has initiated, replication protein A (RPA) binds the single-stranded DNA (ssDNA) overhangs that are produced (Holthausen et al., 2010; Symington and Gautier, 2011). The multimeric RPA filaments on the ssDNA serve to protect the unstable ssDNA from further damage and to facilitate extensive resection by preventing the accumulation of secondary structures on ssDNA (Chen et al., 2013).

Than the recombinase protein Rad51 must displace RPA on the ssDNA and form its own filaments (Figure 1). This process is facilitated, in part, by Rad52 in yeast (Sung, 1997), and BRCA2 and RAD52 in humans (Holloman, 2011). In mammalian cells, the five RAD51 paralogues (RAD51B, RAD51C, RAD51D, XRCC2, XRCC3) are required for RAD51 focus formation (Heyer, 2007). After the DNA ends are resected and Rad51 filaments are formed, a cell is committed to perform HR in order to repair the damaged DNA template (Heyer et al., 2010). Rad51 mediates the search for the homologous DNA sequence and, once the homologous sequence is found, Rad51 filaments facilitate the invasion of the ssDNA overhang into the homologous double-stranded DNA (dsDNA) sequence. Thus, one strand of the duplex DNA is displaced

leaving the complementary strand to serve as a template for repair. This specific recombination structure is referred to as a displacement-loop (D-loop). The invading end of the D-loop can be extended by the DNA polymerase, which would then copy any information that might be missing at the breaksite.

The D-loop represents the branching point for the multiple subpathways of HR (Figure 1). In the absence of a second end, the D-loop may become a full-fledged replication fork in a process termed break-induced replication (BIR). Although this process restores the integrity of the chromosome, it can lead to loss-of-heterozygosity of all genetic information distal to the DSB. In the presence of a second end, the predominant pathway for DSB repair in somatic cells appears to be synthesis-dependent strand annealing (SDSA), in which the extended D-loop is reversed, leading to annealing of the newly synthesized strand with the resected strand of the second end (Pâques and Haber, 1999). This pathway inherently avoids crossovers, which reduces the potential for genomic rearrangements (Figure 1).

Generation of crossovers by double Holliday junction (dHJ) formation is the typical of meiotic recombination but has been recently identified as an intermediate in recombinational DNA repair in vegetative (somatic) cells (Bzymek et al., 2010). dHJ formation involves capture of the second end, a process that is actively blocked by the Rad51 protein in vitro, suggesting an inherent mechanistic bias toward SDSA (Wu et al., 2008). The dHJ intermediate could be resolved by endonucleases in a manner described for the bacterial RuvC protein into crossover or noncrossover products (West, 2003), but the exact mechanisms and identity of proteins involved remain under debate. Alternatively, dHJs can be dissolved by a complex mechanism involving a RecQ-family DNA motor protein (*S. cerevisiae* Sgs1 or human BLM), topoisomerase 3, and cofactors. The two junctions are migrated toward each other, leading to a hemicatenane that is eliminated by Topo3. Genetically, the end point of dissolution is

always a noncrossover, avoiding the potential for rearrangements associated with crossovers (Wu and Hickson, 2003). Crossovers are defined as recombination events that lead to the exchange of flanking markers generating deletions, inversions, or translocations when non-allelic, repeated DNA sequences are involved.

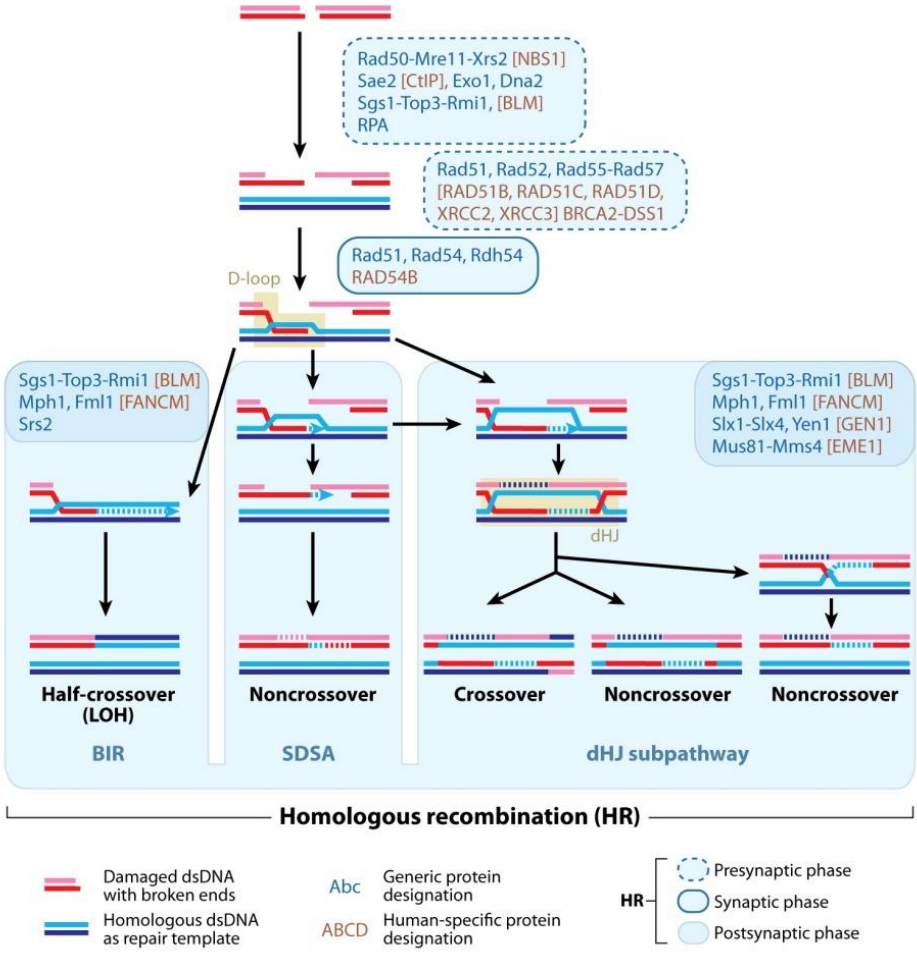

Figure 1 - **HR repair pathways.** Protein names refer to the budding yeast *Saccharomyces cerevisiae* (*blue*). Where different in human, names (*brown*) are given in brackets. For proteins without a yeast homolog, brackets for human proteins are omitted. Broken lines indicate new DNA synthesis and stretches of heteroduplex DNA that upon mismatch repair (MMR) can lead

to gene conversion. Abbreviations: BIR, break-induced replication; dHJ, double Holliday junction; NHEJ, non-homologous end joining; LOH, loss of heterozygosity; SDSA, synthesis-dependent strand annealing; SSA, single-strand annealing. *Adapted from (Heyer et al., 2010).*

Homologous recombination nucleases: Recent genetic studies by several laboratories have elucidated the molecular details of DSB end resection in *Saccharomyces cerevisiae* and a two-step mechanism that includes nucleases and helicases has been proposed (Mimitou and Symington, 2008; Zhu et al., 2008). Shortly after the DSB is formed, the highly conserved Mre11–Rad50–Xrs2 (MRX) complex is recruited to DNA ends to exert architectural and catalytic functions. The *MRE11*, *RAD50*, and *XRS2* genes were originally identified by their requirement for the repair of IR-induced DNA damage and for meiotic recombination in *S. cerevisiae* (Krogh and Symington, 2004). Mre11 and Rad50 are conserved in prokaryotes, archeae, and eukaryotes, whereas Xrs2/Nbs1 is found only in eukaryotes and functions to signal DSBs via the PI3K-like kinase (PIKK), Tel1 in yeast and ATM (ataxia telangiectasia mutated) in mammals (Sharples and Leach, 1995; Stracker and Petrini, 2011). The three proteins interact to form a heterohexameric DNA binding complex containing dimers of each subunit (van der Linden et al., 2009; Williams et al., 2010). The MRX/N complex has several functions in chromosome break metabolism; it is a sensor of DSBs, tethers DNA ends, promotes NHEJ repair, controls 5'-3' resection and is required for telomere maintenance (Stracker and Petrini, 2011). Loss of these functions is tolerated by yeast, but all three genes are essential for cell proliferation in vertebrates (Symington and Gautier, 2011).

The MRX complex provides the Mre11 nuclease which cooperates with Sae2 to catalyze the first step in DSB processing, the removal of a short oligonucleotide from the 5'end (Mimitou and Symington, 2008; Shim et al., 2010). The initial step is essential for meiotic DSB processing, in which DSBs are formed by the topoisomerase

Spo11. Upon DNA cleavage, Spo11 remains covalently attached to the 5' ends of the break presenting a block to resection. Removal of Spo11 from meiotic DSB ends involves a Sae2 and MRX-dependent endonucleolytic step that releases Spo11 bound to a short (10–40 nt) oligonucleotide (Neale et al., 2005) (Figure 2). Several studies suggest that the action of MRX–Sae2 in the initiation of end resection is the rate-limiting step for DSB processing. It was reported that initiation of 5' processing is about three orders of magnitude slower than the 5' processing rate once initiated (Frank-Vaillant and Marcand, 2002). In cells lacking MRX the rate of resection 28 kb away from the break is not significantly different to wild-type cells (Zhu et al., 2008). This result supports a model in which the MRX–Sae2 cleavage step accelerates the rate of resection initiation. The short 3' ssDNA tails formed after MRX–Sae2 cleavage are subject to extensive resection in a second step executed via two parallel pathways (Figure 2). One is dependent on the 5'-3' exonuclease, Exo1, while the other depends on the concerted action of the Sgs1–Top3–Rmi1 complex (STR) with the Dna2 endonucleas. The extensively resected ssDNA tracts formed vary in length from a few hundred nucleotides to tens of kilobases depending on the availability and location of the homologous template and correlate with the kinetics of repair. The formation of long ssDNA tracts might only occur when the preferred template for repair (sister chromatid) is not available, for example, when both sister chromatids are cleaved by the HO endonuclease.

The helicase-nuclease ensemble for DNA resection seems to be a general theme in DNA end-processing machinery. A role for a RecQ family helicase in resection appears to be conserved in human cells and in Xenopus extracts. The mammalian Sgs1 homolog, BLM, functions in a parallel pathway with Exo1 to promote DSB resection (Gravel et al., 2008), while the Xenopus WRN RecQ helicase catalyzes unwinding of

DNA ends followed by 5'–3' degradation of the single-strand tails by the Xenopus DNA2 nuclease (Liao et al., 2008).

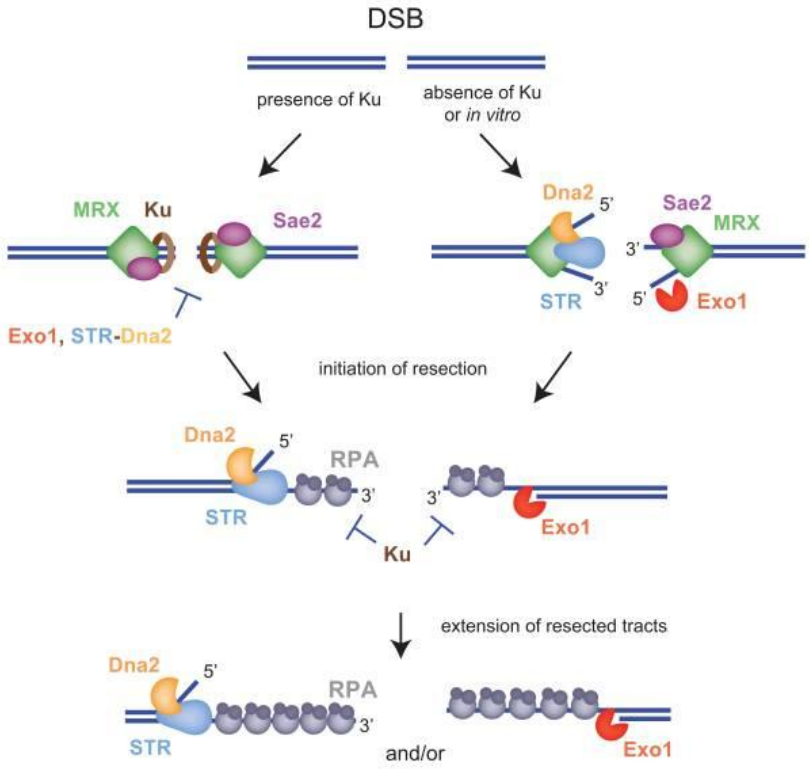

Figure 2 - **Models for the initiation and extension of DSB end resection.** The Mre11–Rad50–Xrs2 (MRX) complex rapidly binds to the DSB to perform a variety of functions including DSB sensing by the checkpoint machinery, tethering of the DSB ends and end-processing in preparation for HR. Genetic studies in *Saccharomyces cerevisiae* suggest that resection initiation requires MRX–Sae2 catalyzed removal of short oligonucleotides from the 5' ends. The intermediate formed is then extensively processed by two parallel pathways dependent on either Sgs1–Top3–Rmi1 (STR) with Dna2 or Exo1. The recent *in vitro* studies confirm the genetic observations but also propose that MRX acts as a scaffold to efficiently recruit the extensive resection machinery. *Adapted from (Mimitou and Symington, 2011).*

Homologous recombination regulation trough cell cycle: One of the most important steps in DSB repair is deciding which specific repair pathway to use. HR uses the genetic information stored in the sister chromatid or in the homologous chromosome to accurately restore lost genetic information at the break site (San Filippo et al., 2008) while non-homologous end joining (NHEJ) directly rejoins two chromosomal ends with no or minimal base pairing at the junction and can generate mutations at the end joining sites (Daley et al., 2005).

In *S. cerevisiae*, the Cdc28 CDK drives directional progress through the cell cycle, dependent on the expression of stage-specific cyclins that modulate CDK activity and impart substrate specificity (Malumbres and Barbacid, 2005; Wohlbold and Fisher, 2009). CDK phosphorylates HR proteins to positively and negatively regulate HR. The availability of sister chromatids largely determines whether HR is a primary pathway, explaining why HR is favored in the S and G2 phases but not in the G0, G1, or M phases (Figure 3).

In haploid *S. cerevisiae* cells, limited end resection can restrict repair of a DSB by HR in the G1 phase of the cell cycle (Aylon et al., 2004; Ira et al., 2004). In yeast, end resection is primarily regulated by CDK-dependent phosphorylation of the Sae2 nuclease (Huertas et al., 2008; Ira et al., 2004) (Figure 3), which determines whether a DSB is channeled into NHEJ or HR. The pivotal phosphorylation occurs at serine 267, located in one of three Sae2 CDK consensus sites (Huertas et al., 2008). An endonuclease mediated DSB at the MAT locus is poorly resected in an *S. cerevisiae sae2Δ* mutant; a *sae2* mutant in which Sae2 serine 267 has been substituted with alanine (*sae2-S267A*) phenocopies the *sae2Δ* strain for unresected DSB ends. In contrast, a Sae2 phosphomimic mutant variant, in which serine 267 has been replaced with aspartic acid (*sae2-S267E*), promotes DSB resection, sidestepping a requirement for CDK activity to sanction DSB resection.

These observations are mirrored by results from human cells, where CtIP, the human homolog of Sae2, is also required for DSB resection (Sartori et al., 2007). Phosphorylation on threonine 847 is required for ssDNA generation and RPA phosphorylation in response to the topoisomerase I inhibitor camptothecin, laser-induced DNA damage, or ionizing radiation (IR) (Huertas and Jackson, 2009).

A transfected phosphomimic CtIP-T847E resects DSBs even after CDK inhibition, whereas the nonphosphorylatable CtIP-T847A mutant impairs resection. CDK phosphorylation of Sae2/CtIP therefore appears to be conserved in eukaryotes as a key switch in determining whether DSB ends are sanctioned for resection and HR. In addition to the conserved mechanism described for *S. cerevisiae* Sae2, human CtIP function also appears to be regulated by an additional CDK phosphorylation at serine 327, a modification that enhances CtIP interaction with the BRCT domain of BRCA1 and is critical for HR (Yu and Chen, 2004; Yun and Hiom, 2009). The function of BRCA1 in HR remains enigmatic. It is interesting to observe that BRCA1 is sumoylated by PIAS1/4 to enhance its ubiquitin ligase activity (Morris et al., 2009) and that CtIP appears to be one of its native ubiquitylation targets (Yu et al., 2006), implying a potential regulatory role of BRCA1 in resection.

Sae2-S267 in *S. cerevisiae* is unlikely to be the exclusive target of CDK relevant to end resection, because the *sae2-S267E* phosphorylation mimic mutation does not completely restore resection to wild-type levels (Huertas et al., 2008). Although Mre11 and Xrs2 have CDK phosphorylation consensus sites, no resection phenotype has been observed when these sites are mutated (Ira et al., 2004). Recent work demonstrated that CDK1 dependent phospohorylation of the Dna2 nuclease at Thr4, Ser17 and Ser237 stimulates its recruitment to DSBs, resection and subsequent Mec1 dependent phosphorylation (Chen et al., 2011). Thus, additional targets regulated to link the HR with cell cycle progression remain to be discover.

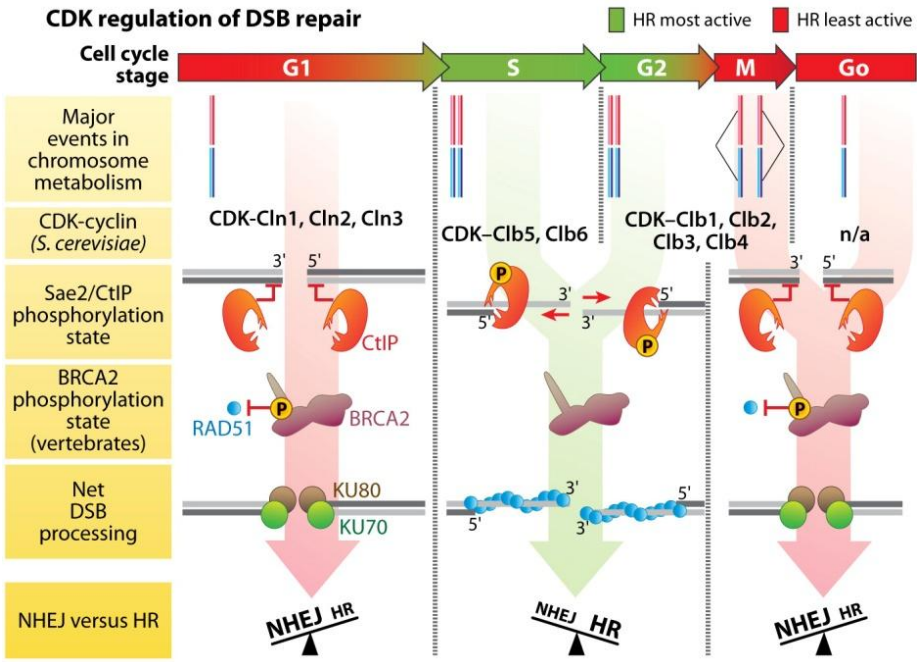

Figure 3 - **Homologous recombination (HR) is regulated by cell-cycle control.** The cell cycle controls the competition between non homologous end joining (NHEJ) and HR in double-strand break (DSB) repair. Cdc28 is the sole cyclin-dependent kinase (CDK) responsible for cell cycle progression in *Saccharomyces cerevisiae*, and partners with the indicated cyclins. In mammals, six CDKs drive cell-cycle progression, and their relative importance varies in different tissue types. *Adapted from (Heyer et al., 2010).*

GENOME STABILITY DURING DNA REPLICATION

Genome duplication is a key event in the life cycle of all proliferating organisms and its careful control is essential to preserve the physical integrity of chromosomes (Arias and Walter, 2007). The main player in this process is the replisome, an assembly of macromolecular machines that serve two essential functions: coupling parental duplex–DNA unwinding with daughter strand synthesis (Macneill, 2012) and integrating DNA damage response signals to modulate fork progression, pausing, and restart (Errico and Costanzo, 2012). Several processes are involved in maintainance of genome integrity during DNA replication by ensuring replisome stability and recovery after fork collapse. Among these mechanism the S phase checkpoint and homologous recombination pathways play a fundamental roles. Checkpoint and HR act synergistically with replisome progression to ensure a faithful completion of DNA replication.

Genome replication: DNA replication requires the coordinated activities of numerous proteins to unpack, copy and repackage the long strands of DNA. During the G1 phase, the origin recognition complex transiently associates with the Cdc6 initiator to recruit a Cdt1-Mcm2-7 heptamer to DNA replication start sites, “origins” (Figure 4a-b; Boos et al., 2012). The end result of this reaction is the formation of a topological link between duplex DNA and two copies of the hexameric Mcm2-7 helicase, which are found tethered via their N-terminal ends. In this configuration, origins are “licensed” for activation; however, the unwinding function of the Mcm2-7 enzyme remains dormant (Remus et al., 2009). Upon entry into S phase, multiple factors are recruited to activate the replication origins by either associating with, or chemically modifying, the Mcm2-7 helicase (Figure 4c; Labib, 2010). According to the current consensus

model, the two Mcm particles are thought to move apart following DNA melting, to travel at the front of the replisome (Botchan and Berger, 2010; Yardimci et al., 2010).
At the core of the replicative machinery, DNA polymerases read the single-stranded DNA (ssDNA) template and insert complementary nucleotides from the cellular nucleotide pool. In eukaryotes, DNA replication is carried out by three distinct DNA polymerases that belong to the B family of DNA polymerases.
DNA polymerase α (Pol α) contains a primase domain that initiates replication by laying down a short RNA primer onto both the leading and lagging strands of the unwound DNA. Pol α then switches to DNA-polymerizing mode and extends these RNA primers by about 20 dNTPs and is subsequently replaced by DNA polymerase δ (Pol δ) or Pol ε. Accumulating evidence suggests that Pol δ is responsible for the replication of the lagging strand (Larrea et al., 2010; Miyabe et al., 2011; Nick McElhinny et al., 2008) in a process that occurs in short stretches known as Okazaki fragments and requires continuous release and rebinding by Pol δ. Pol ε is believed to be responsible for the largely processive and continuous replication of the leading strand (Miyabe et al., 2011; Pursell et al., 2007).
The Pol ε holoenzyme consists of four polypeptides6: Pol2, which contains both polymerase and 3'-5'–exonuclease domains, and three accessory subunits, Dpb2, Dpb3 and Dpb4. In contrast to Pol α and Pol δ, Pol ε is a highly processive enzyme (Chilkova et al., 2007; Núñez-Ramírez et al., 2011; Shcherbakova et al., 2003). Pol δ becomes processive only when bound to proliferating cell nuclear antigen (PCNA). The high fidelity of both Pol ε and Pol δ is in part conferred by their 3'-5'–exonuclease domains responsible to their proofreading activities.

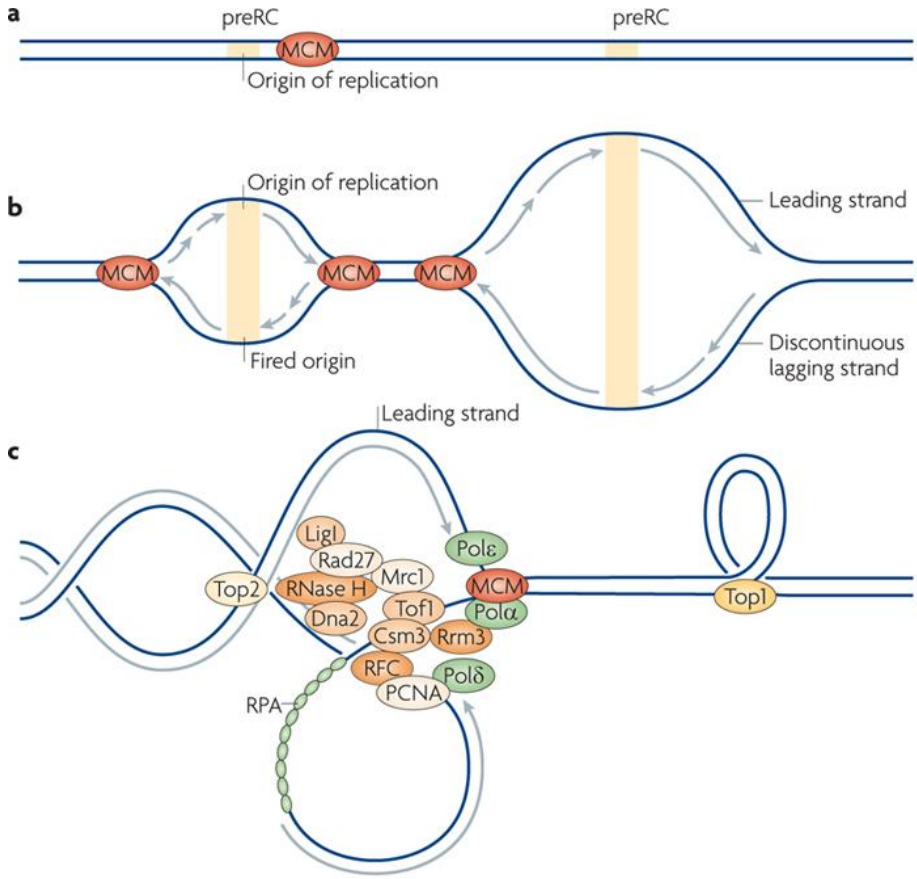

Figure 4 - **Replication initiation and progression. a.** Replication begins from multiple origins, which are marked by the formation of a pre-replicative complex (preRC). **b.** Two replication forks (RFs), which are associated with the replisome that carries out DNA replication, are established at each fired origin. The minichromosome maintenance (MCM) helicase complex is shown ahead of the RFs, unwinding the duplex DNA. Replication is semidiscontinuous: DNA synthesis is continuous on the leading strand and discontinuous on the lagging strand, on which primers are elongated to form Okazaki fragments that are processed and ligated to one another. **c.** Numerous proteins are present at the RF. The MCM helicase unwinds the parental duplex, allowing access to the DNA polymerase-α (Polα) primase, replicative polymerase-δ (Polδ) and polymerase-ε (Polε) (which elongate the primers) and the replication processivity clamp proliferating cell nuclear antigen (PCNA; also known as Pol30), which is loaded by the clamp loader, the replication factor C (RFC) complex. Replication protein A (RPA) binds single-stranded DNA regions exposed at the RF or during lagging-strand synthesis. The discontinuous fragments synthesized on the lagging strand are processed by Rad27 (FEN1 in humans), Dna2 helicase, RNase H, Polδ and DNA ligase I (LigI). Several other factors associate with the RF in

yeast and are represented: DNA topoisomerases 1 (Top1) and Top2, the checkpoint mediators mediator of replication checkpoint protein 1 (Mrc1), Top1-associated factor 1 (Tof1) and chromosome segregation in meiosis protein 3 (Csm3), and the Rrm3 helicase. *Adapted from (Branzei and Foiani, 2010).*

Genome perturbation during replication: the progression of replication forks is hamper at level of DNA lesions occurring under physiological conditions (such as during hydrolysis or metabolism) or induced by external damaging agents (Lindahl, 1993), but also at level of natural impediments such as unusual DNA structures, late replication zones, DNA-binding proteins and transcription units. A common feature of these elements is that they induce the pausing of or completely block the progression of replication forks, increasing the frequency of replication forks breakage events. In all these circumstances it is essential to stabilize the replisome on DNA in order to ensure faithful completion of DNA synthesis once stress conditions are removed.

Hydroxyurea (HU) is among the drugs which affects specifically DNA synthesis. It is an antineoplastic drug, first synthesized in 1869, used in myeloproliferative disorders decreases. HU blocks the production of deoxyribonucleotides (dNTPs) via inhibition of the enzyme ribonucleotide reductase by scavenging tyrosyl free radicals involved in the reduction dNDPs. The depletion of dNTPs result in slow down or block of replication fork progression (Bianchi, 1986).

DNA repeats, such as dinucleotide, trinucleotide, inverted, mirror and direct tandem repeats are among the DNA natural structures that hinder the movement of replisome. That sequences can often undergo structural transitions that lead to the formation of alternative DNA structures, such as cruciforms, triplex H-DNA (DNA structure in which a DNA duplex associates with another DNA single strand in either a parallel or antiparallel orientation) and left-handed Z-DNA which could inhibit replication (Mirkin and Mirkin, 2007). The continuation of DNA synthesis past these elements or the abnormal replication of the repeats has been proposed to lead to

their expansion, and this phenomenon is responsible for many human diseases and hereditary disorders (Mirkin, 2006; Orr and Zoghbi, 2007). Fragile sites are classified as common when they are present in all individuals and rare when they are present in less than 5% of the population. Rare fragile sites arise as a consequence of repeat expansion and have been associated with several human hereditary disorders (Orr and Zoghbi, 2007). By contrast, common fragile sites do not have dinucleotide or trinucleotide repeats, are AT-rich and are normal components of chromosomes that are expressed on the inhibition of DNA replication (Durkin and Glover, 2007). The replication slow zones, that cause slower fork progression, are also thought to represent common fragile sites in yeast (Casper et al., 2002; Cha and Kleckner, 2002). Although there is no AT-rich bias in the replication slow zones, breakage at these sites is stimulated in the absence of an active ataxia telangiectasia and Rad3-related protein (ATR) (Mec1 in *S. cerevisiae*) checkpoint, which is proposed to act by stabilizing the replication forks that are prone to stall at these regions (see below). Other replication-stress-sensitive loci have been reported in yeast and proposed to function analogously with common fragile sites.

In yeast strains with reduced levels of polymerase-α, elevated levels of homologous recombination (HR)-mediated chromosome translocations frequently occur at certain Ty elements (Lemoine et al., 2005). A chromosome region that contains multiple tRNA genes that are known to stall replication forks is also prone to breakage and translocation events, particularly in replication checkpoint mutants (Admire et al., 2006). Exactly what inhibits DNA replication at these fragile elements, leaving unreplicated or single-stranded (ss) DNA regions, is unknown. It might be the unusual conformations that these DNA regions are prone to adopt (Durkin and Glover, 2007). However, secondary structures should no longer be favourable as the replication fork approaches, owing to the positive superhelicity generated in front of the replication

fork. Hairpins or similar structures could form, however, on the lagging-strand template in the time window in which this becomes single stranded, thus interfering with the progression of the lagging-strand polymerase. Preferential instability of repeats on the lagging strand was indeed observed in studies performed on replicating plasmids containing palindromic fragments in both orientations.

S-phase checkpoint: The replication of DNA is a very complex process, that needs to occur accurately, rapidly, and only once per cell cycle in order to prevent genome abnormalities and deleterious loss of genetic information. Hence, problems arising during chromosome replication are inherent to the complexity of the process and a major source of genomic instability. They are aggravated and frequently caused by exogenous environmental agents and reactive metabolic products that constantly damage the DNA, thus generating potential obstacles to the progression of replication forks. In addition, particular regions in the genome constitute a challenge to replication-fork movement and are associated to a high incidence of chromosomal rearrangements. In all these cases, replication forks must maintain their integrity in order to be able to finish chromosome replication accurately when conditions that halt them are eliminated (Friedel et al., 2009; Mimitou and Symington, 2011; Paulsen and Cimprich, 2007). To cope with such situations of replicative stress, eukaryotic cells activate the so called S-phase checkpoint, which detects the replication problems and coordinates a global response to maintain genome integrity (Harrison and Haber, 2006; Zhou and Elledge, 2000).

In budding yeast, the central players of the S-phase checkpoint are Mec1 and Rad53 kinases, which get activated under conditions that threaten DNA replication, such as DNA damage or nucleotide depletion (Branzei and Foiani, 2007; Paulsen and Cimprich, 2007; Tourrière and Pasero, 2007).

The S-phase checkpoint activation requires the establishment of DNA replication forks (Lupardus et al., 2002; Stokes et al., 2002; Tercero et al., 2003) and the generation of ssDNA. The accumulation of ssDNA regions at stalled forks occurs probably because the MCM (*m*inichromosome *m*aintenance *c*omplex) helicase continues DNA unwinding, although uncoupled from DNA synthesis (Byun et al., 2005; Nedelcheva et al., 2005; Sogo et al., 2002). RPA binds the ssDNA and triggers the recruitment of Mec1/ATR at stalled forks by its regulatory subunit, Ddc2/ATRIP (Zou et al., 2003). Mec1 then phosphorylates Mrc1 (the homologue of human Claspin), a component of the replication machinery and a checkpoint mediator that transduces the signal from Mec1 to the effector kinase Rad53 (Alcasabas et al., 2001), which becomes phosphorylated and activated (Figure 5). The S-phase checkpoint response coordinates DNA replication, DNA repair and cell-cycle progression and regulates processes such as firing of replication origins (Santocanale and Diffley, 1998; Shirahige et al., 1998), stabilization of DNA replication forks in response to DNA damage or replicative stress (Lopes et al., 2001; Tercero and Diffley, 2001), resumption of stalled DNA replication forks (Desany et al., 1998; Szyjka et al., 2008), transcriptional induction of DNA damage response genes (Allen et al., 1994), choice of the repair pathway (Kai et al., 2007) and inhibition of mitosis until replication is completed (Allen et al., 1994) (Figure 5).

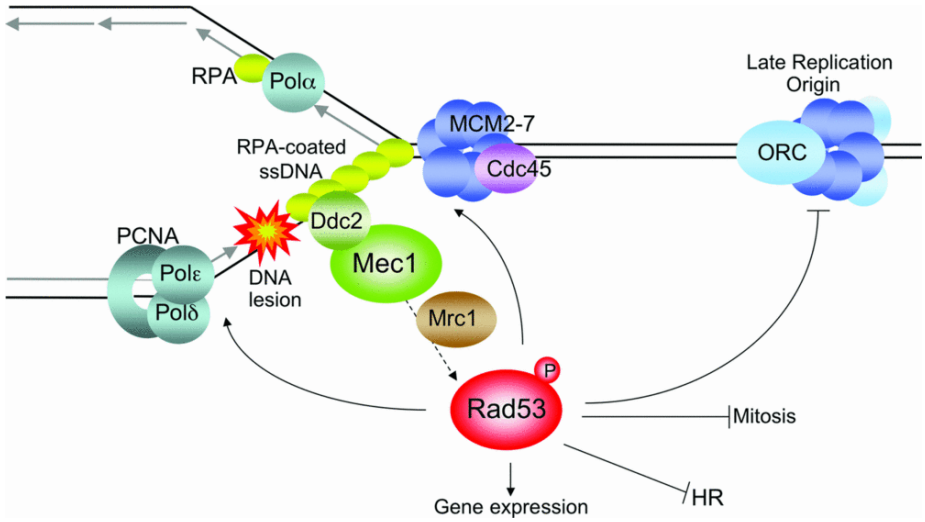

Figure 5 – **S-phase checkpoint.** When replication forks hit DNA lesions or stall because of dNTP deprivation, the helicase and the polymerases may uncouple, exposing regions of ssDNA that cause the activation of the checkpoint response. RPA binds to ssDNA and triggers the recruitment of *S. cerevisiae* Mec1 to the stalled fork by its regulatory subunit Ddc2. Mec1 phosphorylates the mediator Mrc1 and the signal is transduced to the downstream effector kinase Rad53, which is phosphorylated and activated. Rad53 maintains stable, functional DNA replication forks, inhibits firing of late origins, activates gene expression and prevents entry into mitosis and unscheduled recombination. Abbreviations: HR, homologous recombination; PCNA, proliferating cell nuclear antigen. *Adapted from (Segurado and Tercero, 2009).*

Homologous recombination-driven fork recovery: In mammalian cells, agents that trigger the stall or the collapse of replication forks, such as hydroxyurea, thymidine and camptothecin, strongly induce homologous recombination (HR) which promotes the survival to these treatment (Petermann et al., 2010). Replication fork stall or collapse could leads to the formation of DNA gaps or chromosomal rearrangements which are both associated to genomic instability (Branzei and Foiani, 2008; Heller and Marians, 2006; Lopes et al., 2006). Stalled forks and gaps can be recovered by different pathways, including translesion synthesis (TLS), template switching by fork regression, or HR (Branzei and Foiani, 2008) (Figure 6). Although the accuracy of TLS is

lesion- and polymerase-dependent (Prakash et al., 2005), template switching by fork regression and HR is inherently highly accurate. TLS is favored by mono-ubiquitination of proliferating cell nuclear antigen (PCNA) on K164 by the Rad6-Rad18 E2-E3 complex (Figure 6), which enhances the intrinsic affinity of Y-family TLS polymerases (Pol eta) for PCNA through their ubiquitin binding motifs (Prakash et al., 2005). In *S. cerevisiae,* subsequent polyubiquitylation of PCNA by Ubc13-Mms2 (E2) and Rad5 (E3) controls fork regression by a mechanism that is not understood (Prakash et al., 2005). Alternatively, K164 (and K127) can be sumoylated by Ubc9, which leads to recruitment of the Srs2 antirecombinase through its SUMO binding motif (Pfander et al., 2005). As discussed in more detail below, Srs2 dissociates Rad51 from ssDNA, antagonizing Rad51-ssDNA filament formation (Krejci et al., 2003; Veaute et al., 2003). It is unclear whether PCNA ubiquitylation and sumoylation can coexist in a heterotrimeric PCNA ring, and the relationship between HR and these ubiquitylation and sumoylation pathways (Figure 6) is still poorly understood (Branzei et al., 2006, 2008). How is the balance between TLS, fork regression, and HR regulated? Genetic evidence in budding yeast favors the model that TLS and fork regression are primary pathways. At least initially, HR is actively repressed, but the sensitivity of HR mutants to fork stalling agents suggests that this inhibition is temporary. Mutations in *RAD6* or *RAD18* disable TLS and fork regression, leading to severe DNA damage sensitivity. An additional mutation in *SRS2* (Suppressor of Rad Six 2) suppresses the sensitivity to a significant degree by relieving the inhibition of HR (Aboussekhra et al., 1989; Schiestl et al., 1990). These data suggest that Rad6-Rad18 binding to RPA-covered ssDNA (Davies et al., 2008) is kinetically favored over Rad51 filament formation. Possibly, PCNA sumoylation marks a later phase where Srs2 actively removes Rad51 filaments. What regulates PCNA ubiquitylation or sumoylation and whether DDR signaling is involved remain to be determined.

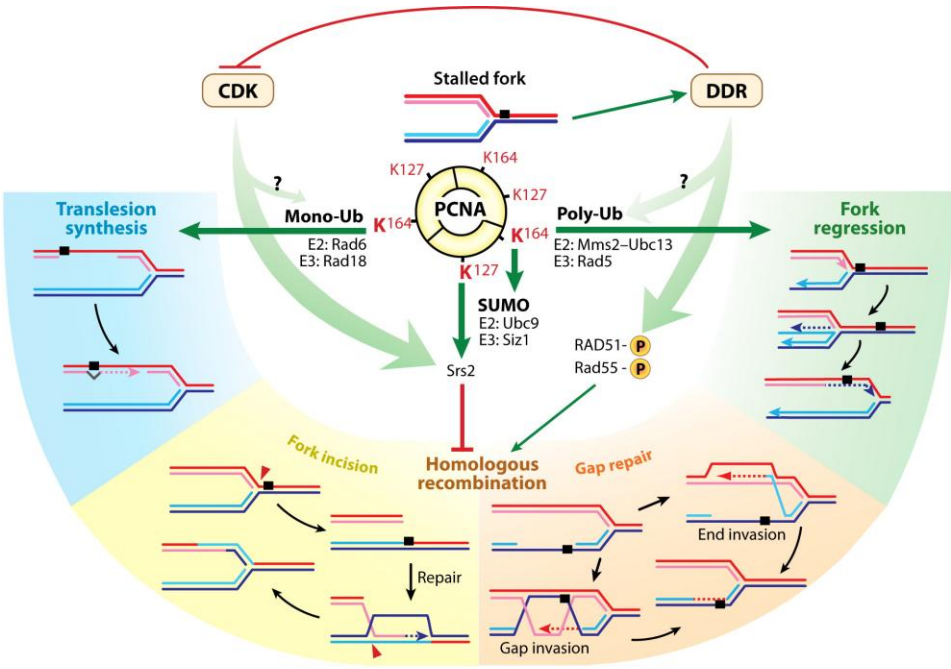

Figure 6 - **Pathways and regulation at stalled replication forks.** Proliferating cell nuclear antigen (PCNA) modification regulates the choice of competing pathways for stalled replication fork recovery. A stalled fork triggers the DNA damage response (DDR), which directly activates homologous recombination (HR). The relationship between the DDR and cell-cycle control to PCNA sumoylation/ubiquitylation has not yet been determined. *Adapted from (Heyer et al., 2010).*

CELLULAR RESPONSE TO OXIDATIVE STRESS

All organisms are exposed to Reactive Oxygen Species (ROS) during the course of normal aerobic metabolism or following exposure to radical-generating compounds (Halliwell, 2006).

Molecular oxygen is relatively unreactive and harmless in its ground state, but can undergo partial reduction to form a number of ROS, including the superoxide anion and hydrogen peroxide (H_2O_2), which can further react to produce the highly reactive hydroxyl radical.

ROS generation naturally arises from environmental insults and from side reactions of normal aerobic metabolism. Mitochondrial respiration is thought to provide the main source of ROS in eukaryotic cells via the process of oxidative phosphorylation (Murphy, 2009). Other metabolic processes can potentially generate endogenous ROS in yeast, include the oxidative protein folding which uses oxygen as a terminal electron acceptor and oxidative peroxisomal fatty acid degradation in the β-oxidation pathway (Hiltunen et al., 2003; Tu and Weissman, 2004).

ROS are toxic agents that can damage a wide variety of cellular components resulting in lipid peroxidation, protein oxidation, and genetic damage through the modification of DNA. An oxidative stress is said to occur when the antioxidant and cellular survival mechanisms are unable to cope with the ROS or the damage caused by them. Various disease processes, including cancer, cardiovascular disease, arthritis, and aging have been shown to involve oxidative damage. *Saccharomyces cerevisiae* responds to an oxidative stress using a number of cellular responses that ensure the survival of the cell following exposure to oxidants. These include defense systems that detoxify ROS, reduce their rate of production, and repair the damage caused by them. Many responses are ROS-specific, but there are also general stress responses that are typically invoked in response to diverse stress conditions.

Oxidative DNA damage: Oxidative DNA damage results from the attack of sugar and base moieties by free radicals and ROS (Cadet et al., 1997). Endogenous oxidative damage is extensive, and the level of steady-state oxidative lesions has been estimated at 10^4-10^5 adducts per cell in mammals, which is equivalent to or higher than estimates of endogenous non-oxidative adducts (Beckman and Ames, 1997; Helbock et al., 1998). Different kinds of DNA lesions are induced by ROS among which DNA single-strand breaks (SSB) and double strand breaks (DSB), base modifications, apurinic/apyrimidinic (AP) sites, and DNA-protein crosslinks (Cadet et al., 1997; Dizdaroglu, 1991). More than 20 different types of oxidatively altered purines and pyrimidines have been detected (Demple and Harrison, 1994; Gajewski et al., 1990). Among them, 8-oxo-7, 8-dihydroguanine (8-oxogua-nine) is the most abundant, and seems to play critical roles in mutagenesis and in carcinogenesis (Fraga et al., 1990; Kasai and Nishimura, 1984). Unlike other oxidative DNA damage, such as thymine glycol and 5', 8-purine cyclodeoxynucleoside (Evans et al., 1993; Kuraoka et al., 2000), 8-oxoguanine does not block DNA synthesis, rather it induces base mispairing. 8-Oxoguanine can pair with both cytosine and adenine during DNA synthesis, and this could lead to GC to TA transversions after two rounds of replication (Grollman and Moriya, 1993). *S. cerevisiae* uses several strategies to prevent 8-oxoG-induced mutagenesis. Base excision repair (BER) has a major role in removing of 8-oxoG from damaged DNA by with Ogg1 glycosylase as a main player (Boiteux et al., 2002; Reagan et al., 1995). Nucleotide excision repair (NER) pathway appears to play a secondary role in the repair of these lesions, whereas recombination and translesion synthesis, mediated by Polη, occur as mechanisms of damage tolerance (Swanson et al., 1999).

This interplay between many pathways suggests that removal of 8-oxoGuanine in DNA and the ROS detoxification in general is critical for yeast.

ROS-specific transcriptional responses: Mounting the defensive response to elevated levels of ROS is a crucial step in preventing cell death from loss of physiologically appropriate redox balance. A key feature in this response is the transcriptional reprogramming of gene expression to provide the requisite changes in proteins to return the redox status of the cell back to an acceptable range. Several, transcriptional regulators that lead to induction of antioxidant proteins have been identified.

Yap1 is the main positive transcriptional regulator involved in the antioxidant response (Harshman et al., 1988). At first it was identified as a functional homologue of mammalian AP-1 capable of conferring a multiple or pleiotropic drug resistance phenotype when overproduced (Hussain and Lenard, 1991; Leppert et al., 1990). Later studies showed that *yap1* null mutants are hypersensitive to oxidative stress demonstrating that it was particularly important in the response to H_2O_2 and diamide (Schnell et al., 1992). Expression analysis of individual genes confirmed that Yap1 regulates the expression of several genes whose products play major roles in the oxidative stress tolerance. These targets include *TRX2* encoding thioredoxin 2 (Kuge and Jones, 1994; Morgan et al., 1997), *TRR1* encoding thioredoxin reductase, and the *TSA1* and *AHP1* both encoded for thioredoxin peroxidases (Charizanis et al., 1999; Lee et al., 1999). The amino-terminus of Yap1 contains a β-Zip DNA binding domain, which is conserved among the AP-1 family of proteins (Moye-Rowley et al., 1989). Yap1 contains two cysteine-rich domain which are critical for Yap1 activation in response to oxidative stresses (Coleman et al., 1999; Morgan et al., 1997). Yap1 activity is mainly regulated by its localization since there is no increase in Yap1 protein levels and only a modest increase in Yap1 DNA-binding activity in response to oxidative stress while the localization changes dramatically (Kuge et al., 1997). Indeed, the protein is relocalized

from cytoplasm into the nucleus in response to an oxidative stress (Coleman et al., 1999; Kuge et al., 1998).

Skn7 is a transcriptional factor that was involved in the oxidative stress tolerance thanks to a genetic screen searching for mutations that cause sensitivity to peroxide (Krems et al., 1996). Mutant strains lacking both Yap1 and Skn7 were no more sensitive to H_2O_2 than either single mutant (Krems et al., 1996; Morgan et al., 1997), suggesting that these two transcriptional regulators act in the same genetic pathway. A likely explanation for this convergence of Yap1 and Skn7 function came from an analysis of transcriptional activation by these factors of promoters of genes involved in thioredoxin homeostasis.

S. cerevisiae contains three different genes encoding thioredoxins, the cytoplasmic *TRX1*, *TRX2* and the mitochondrial *TRX3* (Muller, 1991; Pedrajas et al., 1999). Transcriptional activation of the thioredoxin encoding *TRX2* gene by Yap1 is required for H_2O_2 resistance (Kuge and Jones, 1994). Similarly, *TRX2* is also a target for Skn7 regulation and loss of either Yap1 or Skn7 alone is sufficient to prevent H_2O_2 induction of *TRX2* transcription (Morgan et al., 1997). A simple explanation for these data are that both Yap1 and Skn7 are required for H_2O_2 stimulated *TRX2* expression. This model was directly supported by demonstration that both Yap1 and Skn7 bound to the *TRX2* promoter at different sites (Morgan et al., 1997).

The theme of Yap1 and Skn7 acting at a common promoter to induce oxidative stress tolerance is not restricted to genes influencing thioredoxin-mediated resistance (Lee et al., 1999). A large number of genes are also regulated by Yap1 in a Skn7-independent fashion (Ohtake and Yabuuchi, 1991). While much is known of the molecular basis of Yap1 regulation by oxidative stress, little is known about the control of Skn7 by oxidants. Skn7 is a constitutive nuclear protein and no evidence has

been obtained documenting any changes in the expression of this factor in the presence of oxidants (Raitt et al., 2000).

Thioredoxins system: Thioredoxins (Trxs) are defined as a family of proteins containing a Trx fold and catalyzing oxidoreductase reactions by a dithioldisulphide exchange mechanism involving two redox-active cysteine residues separated by a pair of amino acids (CxxC motif). The Trx fold positions the N-terminal Cys of the catalytic site on the surface in the immediate proximity of the second Cys. This structure allows ready access of the Trx redox centers to the disulfide bridges of associated target proteins. The family of proteins with a Trx fold includes Trxs and glutaredoxins (Grxs), both considered reductants, and disulfide isomerases, regarded as oxidants.

Both thioredoxins and glutaredoxins proteins act by changing the structure and activity of a broad spectrum of target proteins, typically by modifying redox status. Trxs and Grxs are members of families with multiple and partially redundant genes. *S. cerevisiae* encodes a pair of cytosolic Trxs (Muller, 1992), a single Trx reductase and glutathione reductase, two classical Grxs (Gan, 1992), and four monocysteinic Grxs (Figure 7). Single mutants of these genes are viable, but at least one Trx or dicysteinic Grx is necessary for growth (Draculic et al., 2000).

TRX1 and *TRX2* genes encode for *S. cerevisiae* cytoplasmic thioredoxins while *TRR1* encodes for thioredoxin reductase (Gan, 1991). Thioredoxin mutants are auxotrophic for sulfur amino acids, since thioredoxins are the sole hydrogen donors for PAPS reductase, the enzyme that converts 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to sulfite (Muller, 1991). Mutants deleted for TRX1 and TRX2 are also affected in the cell cycle with a prolonged S phase and shortened G1 interval (Muller, 1991). This correlates with the role of cytoplasmic thioredoxins as the major reductants of ribonucleotide reductase during S phase (Camier et al., 2007; Koc et al., 2006). As in

most organisms, yeast thioredoxins are active as antioxidants and play key roles in protection against oxidative stress induced by various ROS (Izawa et al., 1999; Kuge and Jones, 1994). A major part of the antioxidant function of thioredoxins is mediated by peroxiredoxins (Prx's). Oxidized thioredoxins (Trx1/ Trx2) are rapidly observed following exposure to hydrogen peroxide and are detected for 1 hour before returning to the reduced form (Okazaki et al., 2007). Trx2 appears to play the predominant role as an antioxidant, since mutants lacking *TRX2* are hypersensitive to hydroperoxides and mutants containing *TRX2*, in the absence of *TRX1*, show wild-type resistance to oxidative stress (Garrido and Grant, 2002). However, Trx1 and Trx2 appear to be functionally redundant as antioxidants. This is emphasized by the similar redox midpoint potentials (Em) of Trx1 and Trx2 (2275 and 2265 mV, respectively), indicating the interchangeable nature of these proteins (Mason et al. 2006). The differential requirement for Trx1 and Trx2 appears to be related to differences in gene expression; TRX2 expression is strongly upregulated in response to oxidative stress conditions, whereas TRX1 may serve an ancillary or back-up role during conditions in which TRX2 is insufficient to provide an antioxidant defense (Garrido and Grant, 2002).

Yeast also contains a complete mitochondrial thioredoxin system, comprising a thioredoxin (Trx3) and a thioredoxin reductase (Trr2) (Figure 7) (Pedrajas et al., 1999). The redox states of the cytoplasmic and mitochondrial thioredoxin systems are independently maintained and cells can survive in the absence of both systems (Trotter and Grant, 2003). The yeast mitochondrial thioredoxin system has been implicated in protection against oxidative stress generated during respiratory metabolism. However, the mitochondrial thioredoxin reductase was found to have an antioxidant role independent of thioredoxin since mutants deleted for *TRR2* are

sensitive to oxidative stress, compared with *trx3Δ* mutants, which are unaffected in oxidant resistance (Pedrajas et al., 1999; Trotter and Grant, 2003).

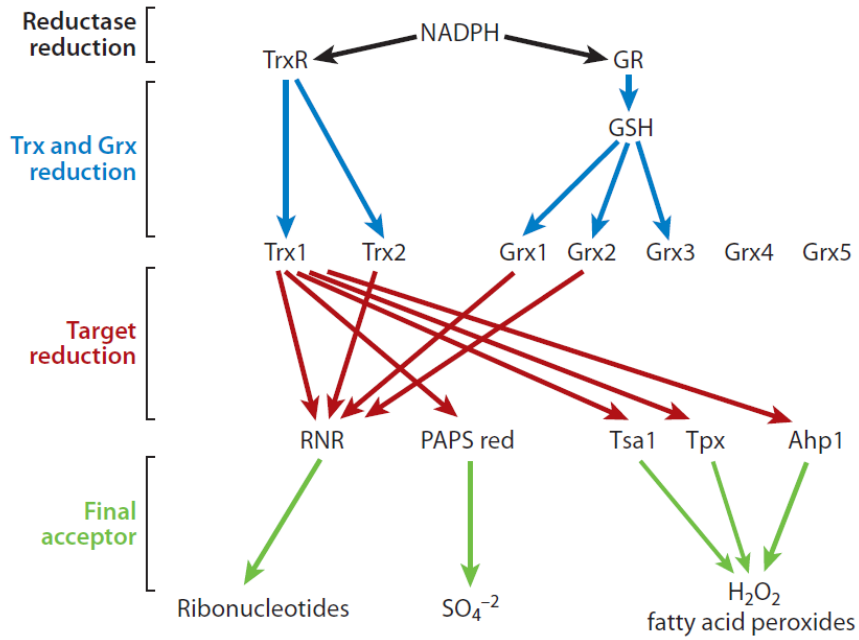

Figure 7 - **Cytoplamic thioredoxins and glutaredoxin systems of *S. cerevisiae*.** *Saccharomyces cereviesie* genome encode for two cytoplasmic thioredoxins (*TRX1* and *TRX2*), a thioredoxin reductase (*TRR1*), a glutathione reductase (GR) and five cytoplasmic glutaredoxins. Thioredoxin (Trx) and glutaredoxin (Grx) are active on different substrates with multiple and partially redundant functions. The number of genes clearly increased with the appearance of multicellular organisms, in part because of new types of Trx and Grx with orthologs throughout the animal and plant kingdoms. *Adapted from (Meyer et al., 2009).*

Hydrogen peroxide during S-phase: Like to other genotoxic agents, eukaryotic genome is more exposed to oxidative stress during DNA replication. Indeed it has been demonstrated that in *S. cerevisiae* exposure to low concentrations of H_2O_2 delays cell cycle progression in G1, S and G2 phases, but that only the delay occurring in S phase is controlled by the DNA damage checkpoints (Leroy et al., 2001).

The mechanisms responsible for the H_2O_2 depending delays occurring in the G1 and G2 phases remain unknown but they could be triggered by cellular reactions other than oxidative attacks on DNA. Several non genotoxic agents have been reported to cause delays in G1 as it happens with heat shock which causes a transient inhibition of Start through decreasing *CLN1* and *CLN2* transcripts (Rowley et al., 1993). A similar mechanism may operate in wild-type cells treated with H_2O_2 in G1.

On the contrary the S-phase delay depends on DNA damage checkpoint activation. One possibility is that H_2O_2 induces more or different DNA damage during S phase because of the intrinsic sensitivity of replicating DNA, or because DNA replication converts primary lesions into DNA structures (DNA breaks, single-stranded DNA or recombination intermediates generated by the stalling of the replication fork) recognizable by DNA damage sensors (Foiani et al., 1998). DNA lesions induced in G1 and G2 by low concentrations of hydrogen peroxide are repaired silently by the BER pathway in wild-type cells and only trigger Rad53 phosphorylation when they are processed incompletely by this pathway (Figure 8, Leroy et al., 2001). Indeed in the absence of Apn1 and Apn2, primary oxidative lesions are converted by glycosylases/AP-lyases into abasic sites with a single strand breaks which are further processed by alternative pathways and recognized by DNA damage checkpoint.

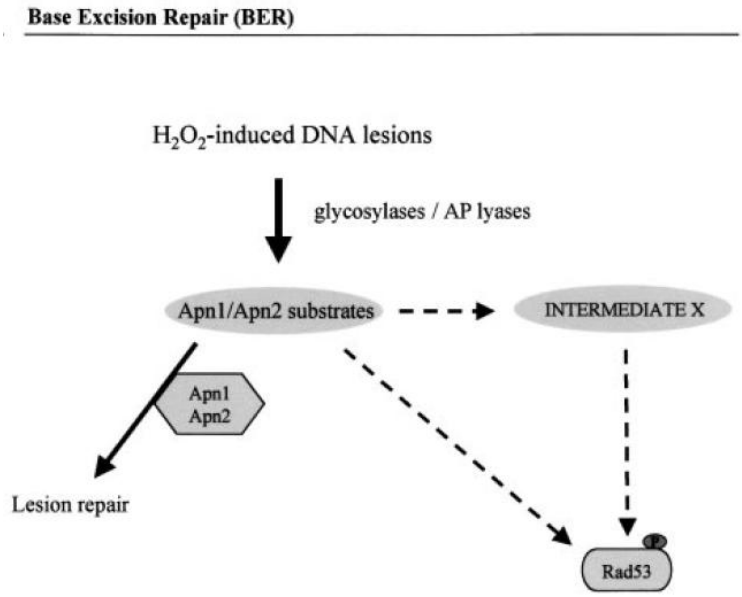

Figure 8 - **Repair of oxidative DNA lesions.** The processing of H_2O_2-induced DNA damage by Apn1/2-dependent pathways (BER) results in its repair and the absence of DNA checkpoint activation during G1 and G2 phases of cell cycle while these lesions trigger Rad53 phosphorylation and checkpoint activation during S-phase or in absence of functional BER. *Adapted from (Leroy et al., 2001).*

RESULTS

During my years of PhD I followed two different projects that I will present in this section of my thesis. Both these projects led us to investigate about various aspects of homologous recombination (HR). HR is a DNA repair pathway which exploits a homologous template for the repair or tolerance of DNA damage and the recovery of stalled or broken replication forks.

The first work that I will present was already started when I began my PhD, thus, my contribution was limited to the experimental part. HR participates in repair of DNA double-strand breaks (DSBs). However HR must be strictly regulated along the cell cycle. It has been demonstrated that Cdks promotes DNA repair by homologous recombination (HR) by promoting the resection of DNA ends. Whether Cdk1 regulates other HR steps was unknown. The aim of this project was to investigate the role of Cdk1 in homology-dependent repair of a DNA double strand break lesion (DSB). The results of this work were published on Plos Genetics journal in the August of 2011.

I personally followed from the beginning the second work that I will present in this thesis. HR has pivotal roles in maintenance of genome integrity also during replication stress. MRX eterotrimeric complex is important for both HR and replisome stability maintenance, indeed its disruptions causes sensitivity to replication inhibitor hydroxyurea (HU). In order to identified factors which cooperate with MRX complex during replication stress we performed a genetic screening searching for spontaneous extragenic mutations that suppress the HU sensitivity of *mre11Δ* cells. We discovered that recessive mutations in thioredoxin reductase (*TRR1*) gene were able to partially suppress the HU sensitivity of *mre11Δ* strain and also that of several mutants defective in the HR machinery.

PLOS GENETICS
vol. 7, no. 8, e1002263, August, 2011
doi: 10.1371/journal.pgen.1002263.

Distinct Cdk 1 requirements during Single-Strand Annealing, Noncrossover, and Crossover Recombination

Camilla Trovesi, Marco Falcettoni, Giovanna Lucchini,
Michela Clerici and Maria Pia Longhese

Dipartimento di Biotecnologie e Bioscienze,
Università di Milano-Bicocca, Milano, Italy.

The lack of Yku70 allows DSB repair by SSA in G1.

HR is inhibited in G1 when Cdk1 activity is low, whereas it occurs during S and G2/M cell cycle phases when Cdk1 activity is high (Aylon *et al.*, 2004; Ira *et al.*, 2004). Although it is well known that Cdk1 promotes resection of DSB ends (Aylon *et al.*, 2004; Ira *et al.*, 2004; Huertas *et al.*, 2008), it is still unclear if other HR steps are regulated by Cdk1. To investigate whether DSB resection is the only step controlled by Cdk1 in HR-mediated DSB repair, we asked if generation of ssDNA at the DSB ends is sufficient to allow HR when Cdk1 activity is low. As DSB resection in G1 is inhibited by the Yku heterodimer and *YKU70* deletion allows ssDNA generation at DSB ends in G1 cells (Clerici *et al.*, 2008), we asked if *yku70Δ* cells are capable to carry out HR in G1.

Homology-dependent repair of a DSB made between tandem DNA repeats occurs primarily by SSA (Jain e*t al.*, 2009), which requires DSB resection and re-annealing of RPA-covered ssDNA by the Rad52 protein (Fishman-Lobell *et al.*, 1992; Pâques and Haber, 1999). This process does not involve strand invasion and is therefore independent of Rad51 (Ivanov *et al.*, 1996). We deleted *YKU70* in a strain where tandem repeats of the *LEU2* gene are 0.7 kb apart and one of them (*leu2::cs*) is adjacent to a recognition site for the HO endonuclease (Figure 5A) (Vaze *et al.*, 2002). The strain also harbors a *GAL-HO* construct that provides regulated *HO* expression. Since homology is restricted to only one DSB end (Figure 9A), the HO-induced break cannot be repaired by gene conversion, making SSA the predominant repair mode. HO was expressed by galactose addition to α-factor-arrested cells that were kept arrested in G1 with α-factor for the subsequent 4 hours. Galactose was maintained in the medium in order to permanently express HO, which can recurrently cleave the HO sites eventually reconstituted by NHEJ-mediated DSB repair. Kinetics of DSB repair was evaluated by Southern blot analysis with a *LEU2* probe that also allowed following 5'-end resection on each side of the break by monitoring the disappearance

of the HO-cut DNA bands. The quality and persistence of the cell cycle arrest was assessed by FACS analysis (Figure 9B) and by measuring Cdk1 kinase activity (Figure 9F). Consistent with the requirement of Cdk1 activity for DSB resection and repair, both the 1.8 kb and 3.2 kb HO-cut band signals remained high throughout the experiment in wild type G1 cells (Figure 9C, D), where the 2.9 kb SSA repair product was only barely detectable (Figure 9C, E). By contrast, the SSA repair product accumulated in *yku70Δ* G1 cells (Figure 5C, E), where both the 1.8 kb and 3.2 kb HO-cut band signals decreased (Figure 5C, D). The ability of *yku70Δ* cells to carry out SSA does not require Cdk1. In fact, Cdk1 activity, which was present in exponentially growing wild type and *yku70Δ* cells, dropped to undetectable levels after G1 arrest (time 0) and remained undetectable in both cultures throughout the experiment (Figure 9F). Thus, the lack of Yku allows DSB repair by SSA in G1, suggesting that ssDNA generation is sufficient to bypass Cdk1 requirement for SSA.

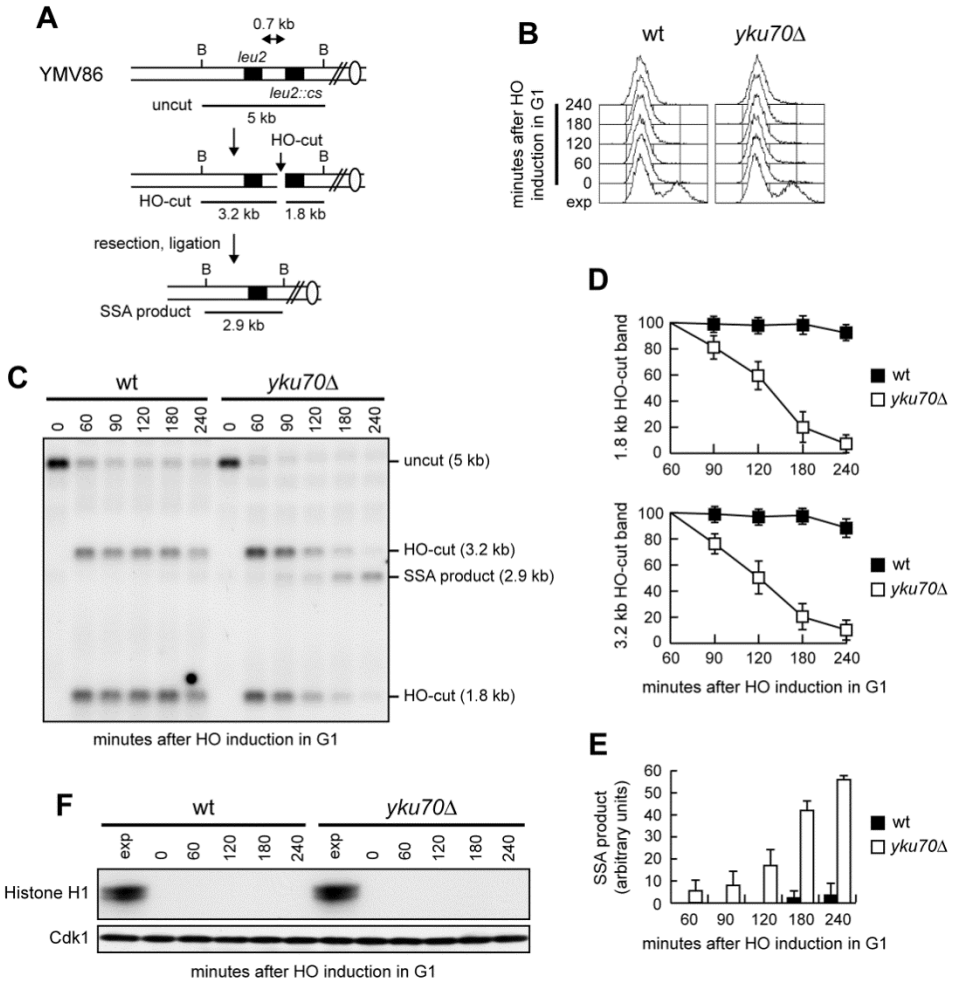

Figure 9 – **SSA-mediated DSB repair in *yku70Δ* cells.** (A) Map of the YMV86 chromosome III region where the HO-cut site is flanked by homologous *leu2* sequences that are 0.7 kb apart. HO-induced DSB formation results in generation of 3.2 kb and 1.8 kb DNA fragments (HO-cut) that can be detected by Southern blot analysis of BglII-digested genomic DNA with a *LEU2* probe. DSB repair by SSA generates a product of 2.9 kb (SSA product). B, BglII. (B-E) Exponentially growing YEP+raf (exp) cell cultures of wild type YMV86 and its *yku70Δ* derivative strain were arrested in G1 with α-factor (time zero) and transferred to YEP+raf+gal in the presence of α-factor. (B) FACS analysis of DNA content. (C) Southern blot analysis of BglII-digested genomic DNA. (D, E) Densitometric analysis of the HO-cut (D) and the SSA (E) band signals. Plotted values are the mean value ±SD from four independent experiments as in (C), enclosing that described in (F). The intensity of each band was normalized with respect to a

loading control. (F) YMV86 derivative strains with the indicated genotypes and expressing fully functional Cdc28-HA were treated as in (B-E). Cell samples were collected at the indicated times to assay Cdk1 kinase activity in anti-HA immunoprecipitates by using histone H1 as substrate (top row) and to determine Cdk1 levels by western blot analysis with anti-HA antibody (bottom row).

SSA-based DNA repair requires degradation of the 5' DSB ends to reach the complementary DNA sequences that can then anneal. If SSA in *yku70Δ* G1 cells depends on generation of 3'-ended ssDNA at DSB ends, then failure of resection to reach the homologous distal *leu2* sequence should prevent SSA. Interestingly, Cdk1-independent resection takes place in *yku70Δ* cells, but it is confined to DNA regions closed to the DSB site (Clerici *et al.*, 2008), suggesting that other proteins limit extensive DSB resection in the absence of Yku. We therefore asked whether increasing the distance between the complementary *leu2* sequences prevented DSB repair by SSA in *yku70Δ* G1 cells. To this end, we monitored SSA-mediated repair of an HO-induced DSB in a strain where the donor *leu2* sequence was positioned 4.6 kb away from the HO recognition site at *leu2::cs* (Figure 10A) (Vaze *et al.*, 2002). HO expression was induced in α-factor-arrested cells that were kept blocked in G1 with α-factor in the presence of galactose (Figure 10B). Consistent with previous findings (Clerici *et al.*, 2008), resection in *yku70Δ* G1 cells was restricted to DNA regions closed to the break site. In fact, the 2.5 kb HO-cut signal decreased more efficiently in *yku70Δ* than in wild type G1 cells, whereas similar amounts of the 12 kb HO-cut signal were detectable in both wild type and *yku70Δ* G1 cells (Figure 10C, D). Thus, 5'-3' nucleolytic degradation in *yku70Δ* G1 cells failed to proceed beyond the distal *leu2* hybridization region. The inability of resection to uncover the homologous distal *leu2* sequence prevented DSB repair by SSA in *yku70Δ* G1 cells. In fact, the 8 kb SSA repair product was only barely detectable in both wild type and *yku70Δ* G1 cells throughout the experiment (Figure 10C, E). By contrast, when a similar experiment was

performed in G2-arrested cells (Figure 10B), where the inhibitory function of Yku on DSB resection is relieved (Bonetti *et al.*, 2010; Shim *et al.*, 2010), the 8 kb SSA repair product was clearly detectable in wild type and *yku70Δ* cells (Figure 10C, E), which both showed also a decrease of the 12 kb HO-cut signals compared to the same strains arrested in G1 (Figure 10C, D). Thus, the ability of *yku70Δ* G1 cells to repair a DSB by SSA depends on the extent of resection.

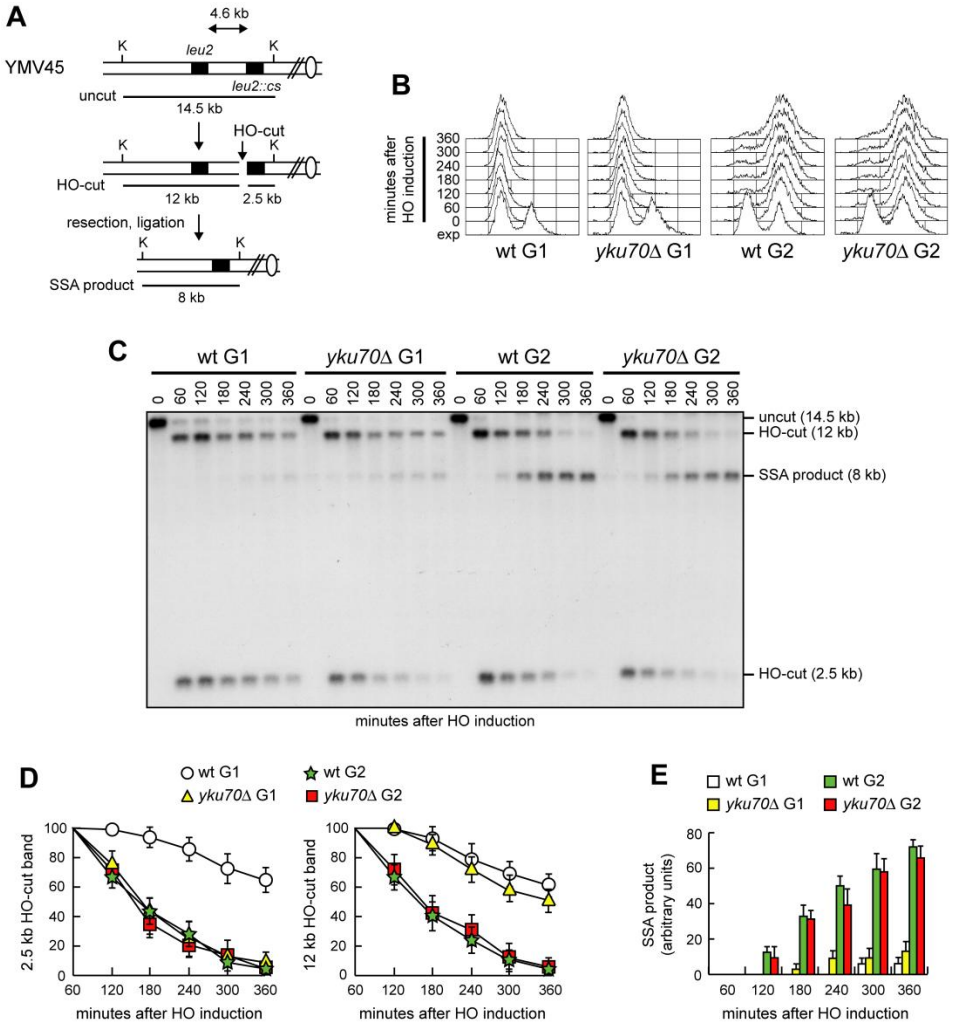

Figure 10 - **SSA-mediated DSB repair in *yku70Δ* G1 and G2 cells.** Map of the YMV45 chromosome III region where the HO-cut site is flanked by homologous *leu2* sequences that are 4.6 kb apart. HO-induced DSB formation results in generation of 12 kb and 2.5 kb DNA fragments (HO-cut) that can be detected by Southern blot analysis of KpnI-digested genomic DNA with a *LEU2* probe. DSB repair by SSA generates a product of 8 kb (SSA product). K, KpnI. (B-E) Exponentially growing YEP+raf (exp) cell cultures of wild type YMV45 and its *yku70Δ* derivative strain were arrested at time zero in G1 with α-factor or in G2 with nocodazole and transferred to YEP+raf+gal in the presence of α-factor or nocodazole, respectively. (B) FACS analysis of DNA content. (C) Southern blot analysis of KpnI-digested genomic DNA. (D, E) Densitometric analysis of the HO-cut (D) and the SSA (E) band signals. Plotted values are the mean value ±SD from three independent experiments as in (C). The intensity of each band was normalized with respect to a loading control.

If ssDNA generation were the limiting step in SSA-mediated DSB repair in G1, then increasing the efficiency/extent of resection should enhance the ability of *yku70Δ* cells to carry out SSA in G1. The lack of the checkpoint protein Rad9 has been shown to allow DSB resection in G2 cells that displayed low Cdk1 activity due to high levels of the Cdk1 inhibitor Sic1 (Lazzaro *et al.*, 2008). Thus, we asked whether the lack of Rad9 enhanced the efficiency of DSB resection in *yku70Δ* G1 cells. To compare resection efficiency independently of DSB repair, we monitored the appearance of the resection products at an HO-induced DSB generated at the *MAT* locus (Figure 11B) of G1-arrested (Figure 11A) cells, which were not able to repair this DSB because they lacked the homologous donor sequences *HML* and *HMR* (Lee *et al.*, 1998). As expected, wild type cells showed very low levels of the 3'-ended resection products (r1 to r5), which instead clearly accumulated in both *yku70Δ* and *yku70Δ rad9Δ* cells (Figure 11C, D). Moreover, the longest r4 and r5 resection products were detectable in *yku70Δ rad9Δ* cells 120 minutes earlier than in *yku70Δ* cells (Figure 11C, D), indicating that the lack of Rad9 enhances the resection efficiency of *yku70Δ* G1 cells. Interestingly, although *RAD9* deletion was shown to allow MRX-dependent ssDNA generation in Sic1 overproducing G2 cells (Lazzaro *et al.*, 2008), *rad9Δ* G1 cells did not show increased efficiency of DSB resection compared to wild type cells (Figure 11C,

D). Thus, Rad9 limits extensive resection in *yku70Δ* cells, but its lack is not sufficient, by itself, to escape the inhibitory effect of Yku on DSB resection in G1.

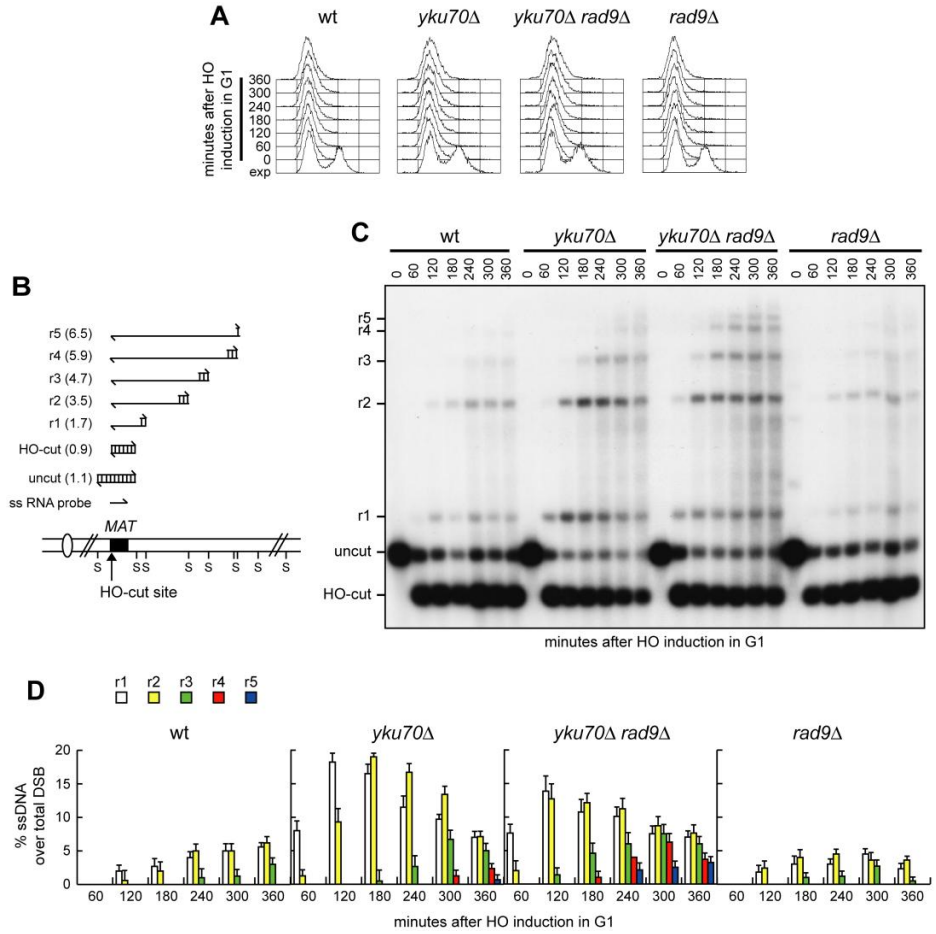

Figure 11 - **Rad9 inhibits extensive DSB resection in yku70D G1 cells.** Exponentially growing YEP+raf (exp) cell cultures of wild type JKM139 and its derivative mutant strains were arrested in G1 with α-factor (time zero) and transferred to YEP+raf+gal in the presence of α-factor. (A) FACS analysis of DNA content. (B) System used to detect DSB resection. Gel blots of SspI-digested genomic DNA separated on alkaline agarose gel were hybridized with a single-stranded *MAT* probe specific for the unresected strand. 5'-3' resection progressively eliminates SspI sites (S), producing larger SspI fragments (r1 through r5) detected by the

probe. (C) Analysis of ssDNA formation as described in (B). (D) Densitometric analysis of the resection products. Plotted values are the mean value ±SD from three independent experiments as in (C). See Material and Metods for details.

The lack of Rad9 enhances resection in *yku70Δ* cells.

Because DSB resection in G1 was more efficient in *yku70Δ rad9Δ* cells than in *yku70Δ* cells, we asked whether the lack of Rad9 allows efficient SSA-mediated DSB repair in *yku70Δ* G1 cells carrying tandem repeats of the *LEU2* gene 4.6 kb apart. Indeed, the amount of SSA repair products in G1 was much higher in *yku70Δ rad9Δ* cells than in wild type, *yku70Δ* or *rad9Δ* cells (Figure 12A, C). Consistent with DSB resection being more extensive in *yku70Δ rad9Δ* than in *yku70Δ* G1-arrested cells (Figure 11), the decrease of the 12 kb HO-cut band signal was much more apparent in *yku70Δ rad9Δ* than in *yku70Δ* G1 cells, whereas the 2.5 kb HO-cut band signal decreased with similar kinetics in both G1 cell cultures (Figure 12B, 8D). Cdk1 kinase activity, which was present in all exponentially growing cells, was not required for accumulation of the repair products in *yku70Δ rad9Δ* cells, as it was undetectable in all G1-arrested cell cultures throughout the experiment (Figure 12E).

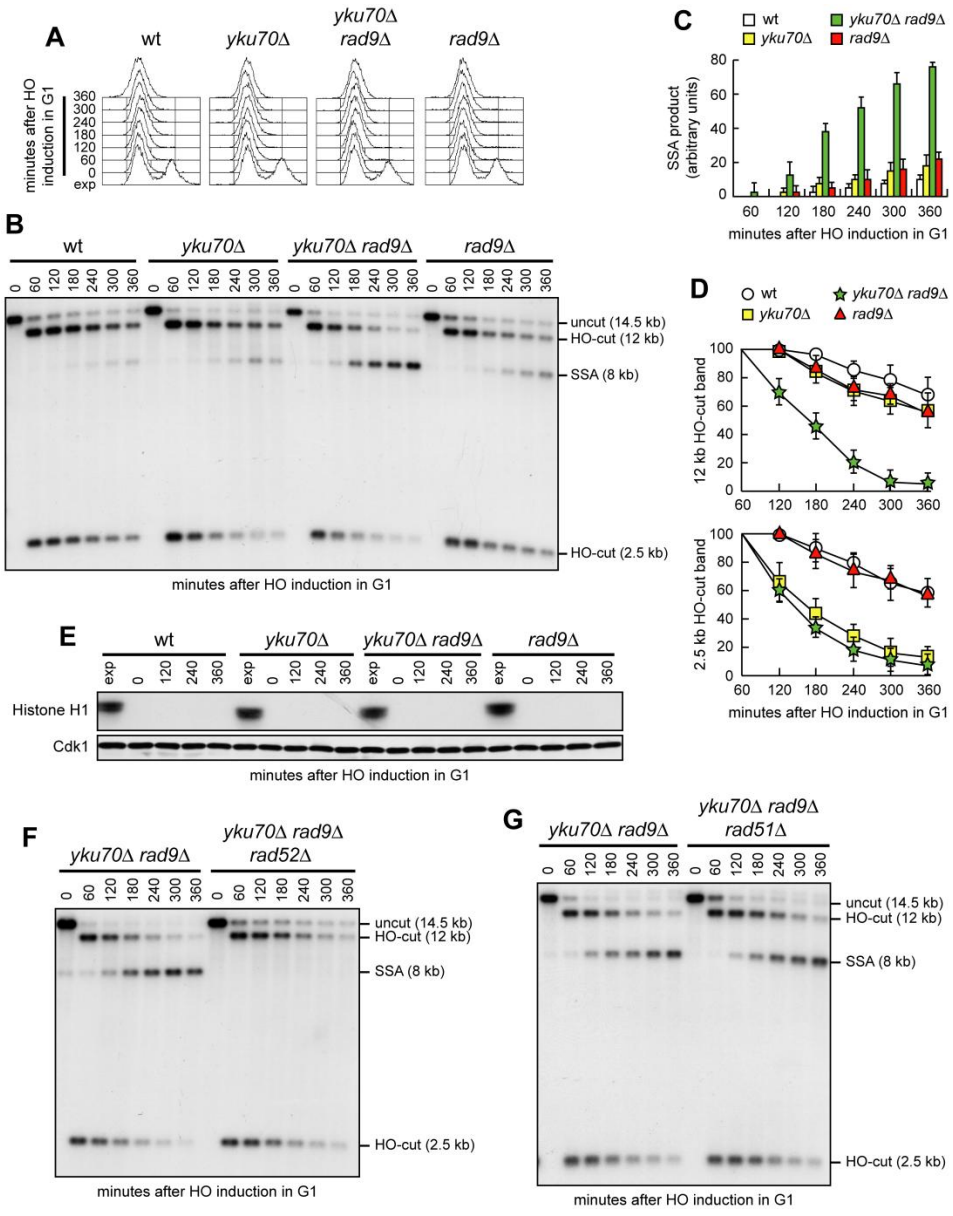

Figure 12 – ***RAD9* deletion increases SSA efficiency in *yku70Δ* G1 cells.** (A-D) Exponentially growing YEP+raf (exp) cell cultures of wild type YMV45 and its derivative mutant strains were arrested in G1 with α-factor (time zero) and transferred to YEP+raf+gal in the presence of α-factor. (A) FACS analysis of DNA content. (B) DSB repair by SSA was analyzed as described in Figure 14 (C, D) Densitometric analysis of the SSA (C) and the HO-cut (D) band signals. Plotted

values are the mean value ±SD from four independent experiments as in (B), enclosing that described in (E). (E) YMV45 derivative strains with the indicated genotypes and expressing fully functional Cdc28-HA were treated as in (A-D). Cell samples were taken at the indicated times to assay Cdk1 kinase activity (top row) and to determine Cdk1 levels (bottom row) as in Figure 13F. (F, G) Exponentially growing YEP+raf cell cultures of YMV45 derivative strains were arrested in G1 with α-factor (time zero) and transferred to YEP+raf+gal in the presence of α-factor. DSB repair by SSA was analized as described in Figure 10.

SSA requires the strand-annealing activity of the Rad52 protein, but it occurs independently of Rad51 (Ivanov *et al.*, 1996). Consistent with the SSA repair mode, formation of the repair products in G1-arrested *yku70Δ rad9Δ* cells was abolished by *RAD52* deletion (Figure 12F), whereas it was unaffected by *RAD51* deletion (Figure 12G). As a DSB flanked by direct repeats could be repaired, at least in principle, also by Rad51-dependent BIR (Jain *et al.*, 2009), the finding that *yku70Δ rad9Δ* and *yku70Δ rad9Δ rad51Δ* G1 cells accumulated the 8 kb repair product with similar kinetics (Figure 12G) indicates that SSA is responsible for this repair event. Thus, we conclude that the lack of Rad9 increases the ability of *yku70Δ* cells to carry out DSB repair by SSA in G1, likely by enhancing the efficiency of DSB resection.

If competence for SSA-mediated DSB repair relies solely on 3'-ended ssDNA generation, then this repair process should take place with similar efficiency in G1- and G2-arrested *yku70Δ rad9Δ* cells. As this expectation is based on the assumption that G1- and G2-arrested *yku70Δ rad9Δ* cells resect DSB ends with similar efficiencies, we compared resection (Figure 13 B, C) and SSA (Figure 13B, D) in *yku70Δ rad9Δ* cells arrested either in G1 or in G2 (Figure 13A) during break induction. Disappearance of the 2.5 kb and 12 kb HO-cut bands occurred with similar kinetics in G1- and G2-arrested *yku70Δ rad9Δ* cells (Figure 13B, C), which also accumulated similar amounts of the 8 kb SSA repair product (Figure 13B, D). As expected, Cdk1 kinase activity was undetectable in *yku70Δ rad9Δ* cells during the α-factor arrest, whereas it was high in

nocodazole-arrested G2 cells (Figure 13E). Thus, DSB resection is the limiting step in DSB repair by SSA.

If SSA is generally restricted to G2 only because high Cdk1 activity allows DSB resection, then inactivation of Cdk1 in G2 should prevent SSA in wild type but not in *yku70Δ rad9Δ* cells, where DSB resection occurs independently of Cdk1. Thus, we compared DSB repair by SSA in G2-arrested wild type and *yku70Δ rad9Δ* cells expressing high levels of a stable version of the mitotic Clb-Cdk1 inhibitor Sic1 (Sic1nt*Δ*) (Desdouets *et al.*, 1998). Consistent with the hypothesis that Cdk1 promotes SSA by regulating the resection step, Sic1 overproduction inhibited SSA repair in G2-arrested wild type cells but not in *yku70Δ rad9Δ* cells. In fact, the 8 kb SSA repair product accumulated in *yku70Δ rad9Δ GAL-SIC1ntΔ* cells (Figure 13F, G), which showed a decrease of both the 2.5 kb and 12 kb HO-cut band signals (Figure 13F, H). By contrast, the same repair product was only barely detectable in G2-arrested *GAL-SIC1ntΔ* cells, where the HO-cut band signals remained high throughout the experiment (Figure 13F, H).

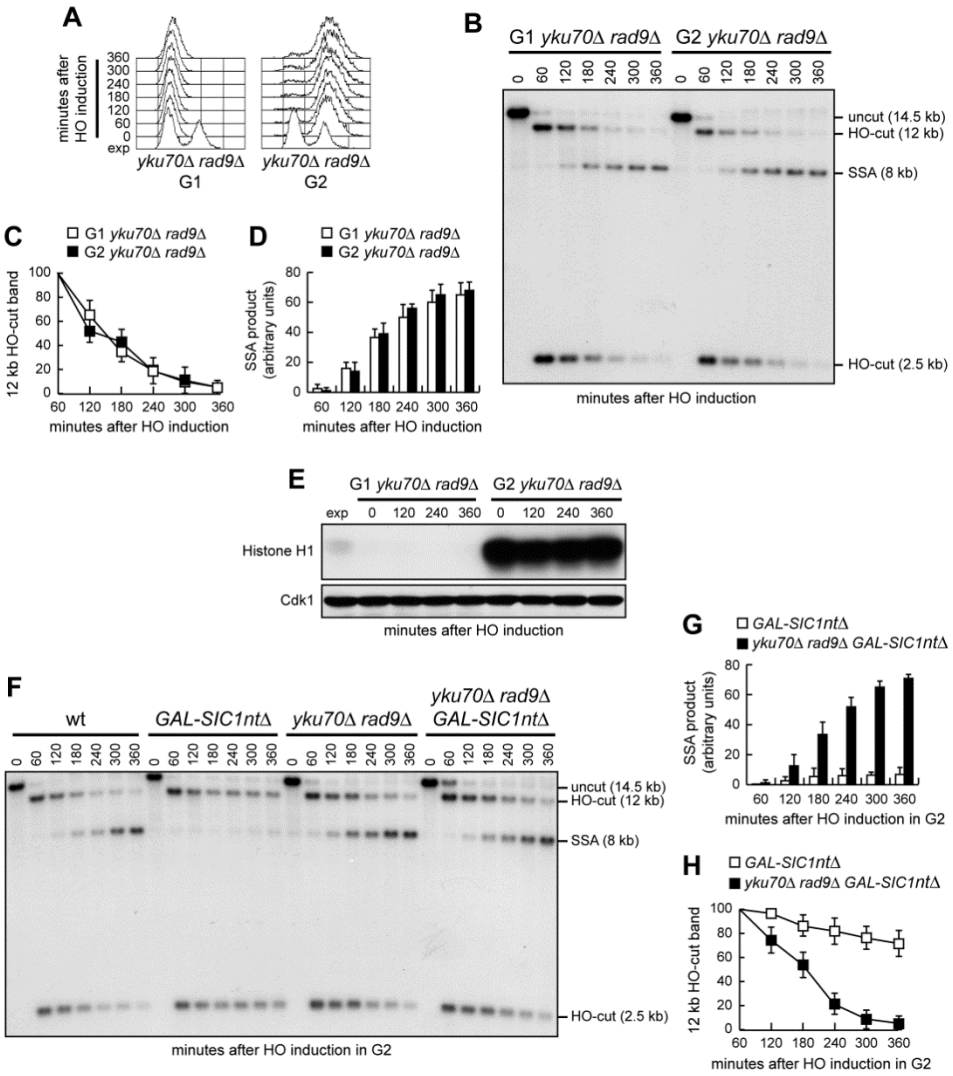

Figure 13 – **DSB resection is the limiting step in DSB repair by SSA.** (A-D) Exponentially growing YEP+raf (exp) YMV45 *yku70Δ rad9Δ* cells were arrested in G1 with α-factor or in G2 with nocodazole and transferred to YEP+raf+gal in the presence of α-factor or nocodazole, respectively. (A) FACS analysis of DNA content. (B) DSB repair by SSA was analyzed as described in Figure 10. (C, D) Densitometric analysis of the 12 kb HO-cut (C) and 8 kb SSA (D) band signals. Plotted values are the mean value ±SD from four independent experiments as in (B), enclosing that described in (E). The intensity of each band was normalized with respect to a loading control. (E) YMV45 *yku70Δ rad9Δ* cells expressing fully functional Cdc28-HA were treated as in (A-D). Cell samples were taken at the indicated times to assay Cdk1 kinase activity (top row) and to determine Cdk1 levels (bottom row) as in Figure 9F. (F-H) Exponentially

growing YEP+raf YMV45 derivative cells with the indicated genotypes were arrested at time zero in G2 with nocodazole and transferred to YEP+raf+gal in the presence of nocodazole. Cell cycle arrest was verified by FACS analysis (not shown). (F) DSB repair by SSA was analyzed as described in Figure 10. (G, H) Densitometric analysis of the 8 kb SSA (G) and 12 kb HO-cut (H) band signals. Plotted values are the mean value ±SD from three independent experiments as in (F). The intensity of each band was normalized with respect to a loading control.

The lack of Yku70 allows noncrossover recombination in G1. When both ends of a DSB share homology with an intact DNA sequence, repair by Rad51-dependent recombination pathways leads to the formation of noncrossover or crossover products. We investigated whether generation of 3'-ended ssDNA can bypass Cdk1 requirement also in this process. To detect crossovers and noncrossovers at the molecular level, we used a haploid strain that bears two copies of the *MATa* sequence (Figure 14A) (Prakash *et al.*, 2009; Saponaro *et al.*, 2010). One copy is located ectopically on chromosome V and carries the recognition site for the HO endonuclease, while the endogenous copy on chromosome III carries a single base pair mutation that prevents HO recognition (*MATa-inc*). Upon galactose addition, the HO-induced DSB can be repaired by Rad51-dependent HR using the uncleavable *MATa-inc* sequence as a donor. This repair event can occur either with or without an accompanying crossover (Figure 14A) with the proportion of crossovers being 5–6% among the overall repair events (Prakash *et al.*, 2009; Saponaro *et al.*, 2010). We induced HO expression in α-factor-arrested cells that were kept arrested in G1 in the presence of galactose (Figure 14B). Galactose was maintained in the medium to cleave the HO sites that were eventually reconstituted by NHEJ-mediated DSB repair. The 3 kb *MATa* band resulting from recombination events that are not associated to crossovers re-accumulated in both *yku70Δ* and *yku70Δ rad9Δ* G1 cells, but not in wild type and *rad9Δ* G1 cells (Figure 14C, 14D). The repair efficiency in both *yku70Δ* and *yku70Δ rad9Δ* G1 cells was around 40% after 8 hours (Figure 14C, 14D), reaching 80–

90% after 24 hours (data not shown). This finding indicates that the absence of Yku is sufficient for noncrossover HR events to take place despite of the low Cdk1 activity.

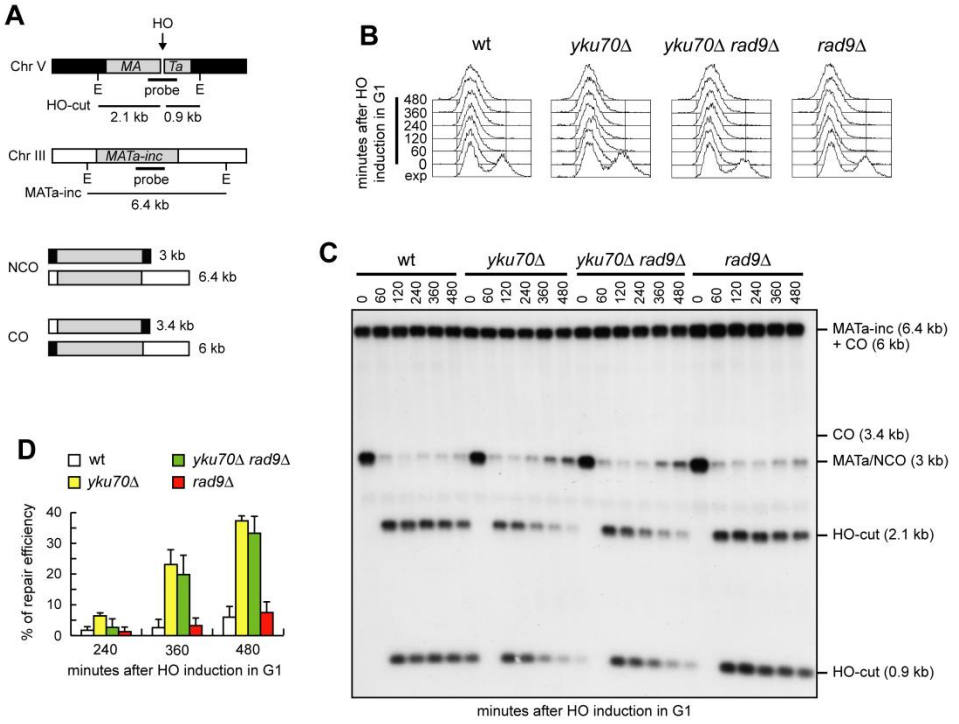

Figure 14 – **Generation of ssDNA bypasses Cdk1 requirement for noncrossover recombination.** (A) In all the strains with the indicated genotypes, galactose-induced HO generates a DSB at a *MATα* DNA sequence inserted on chromosome V, while the homologous *MATα-inc* region on chromosome III cannot be cut by HO and is used as a donor for HR-mediated repair, which can generate both noncrossover (NCO) and crossover (CO) products. The sizes of EcoRI (E) fragments detected by the depicted probe are indicated. (B-D) Exponentially growing YEP+raf (exp) cell cultures were arrested in G1 with α-factor (time zero) and transferred to YEP+raf+gal in the presence of α-factor. (B) FACS analysis of DNA content. (C) Southern blot analysis of EcoRI-digested genomic DNA with the *MATα* probe depicted in A. (D) Densitometric analysis of the repair signals. Plotted values are the mean value ±SD from three independent experiments as in (C). See material and methods for details.

Cdk1 requirement for crossover recombination. Interestingly, the 3.4 kb chromosomal band expected in the experiment above in case of crossover products

was not detectable in any G1 cell culture (Figure 14C), suggesting a role for Cdk1 in promoting crossover outcomes that is different from its function in DSB resection. We then compared the products of interchromosomal recombination in G1- and G2-arrested wild type and *yku70Δ rad9Δ* cells (Figure 15A). As expected, Cdk1 kinase activity remained undetectable in all α-factor arrested cell cultures, whereas it was high in G2-arrested cells (Figure 15B). The overall DSB repair efficiency of G1-arrested *yku70Δ rad9Δ* cells was similar to that of G2-arrested wild type and *yku70Δ rad9Δ* cells (Figure 15C and 15D). However, while no crossover events were detectable in *yku70Δ rad9Δ* G1 cells, ∼4–5% of repair events were associated to crossovers in both wild type and *yku70Δ rad9Δ* G2 cells, as indicated by the appearance of the 3.4 kb crossover band (Figure 15C and 15E). Thus, *yku70Δ rad9Δ* G1 cells appear to be specifically defective in generating crossover products. This inability was not due to the absence of Yku and/or Rad9, because similar amounts of crossover products were detectable in wild type and *yku70Δ rad9Δ* G2-arrested cells (high Cdk1 activity) (Figure 15C and 15E). These results suggest that Cdk1 has a function in promoting crossover recombination that is independent of its role in DSB resection.

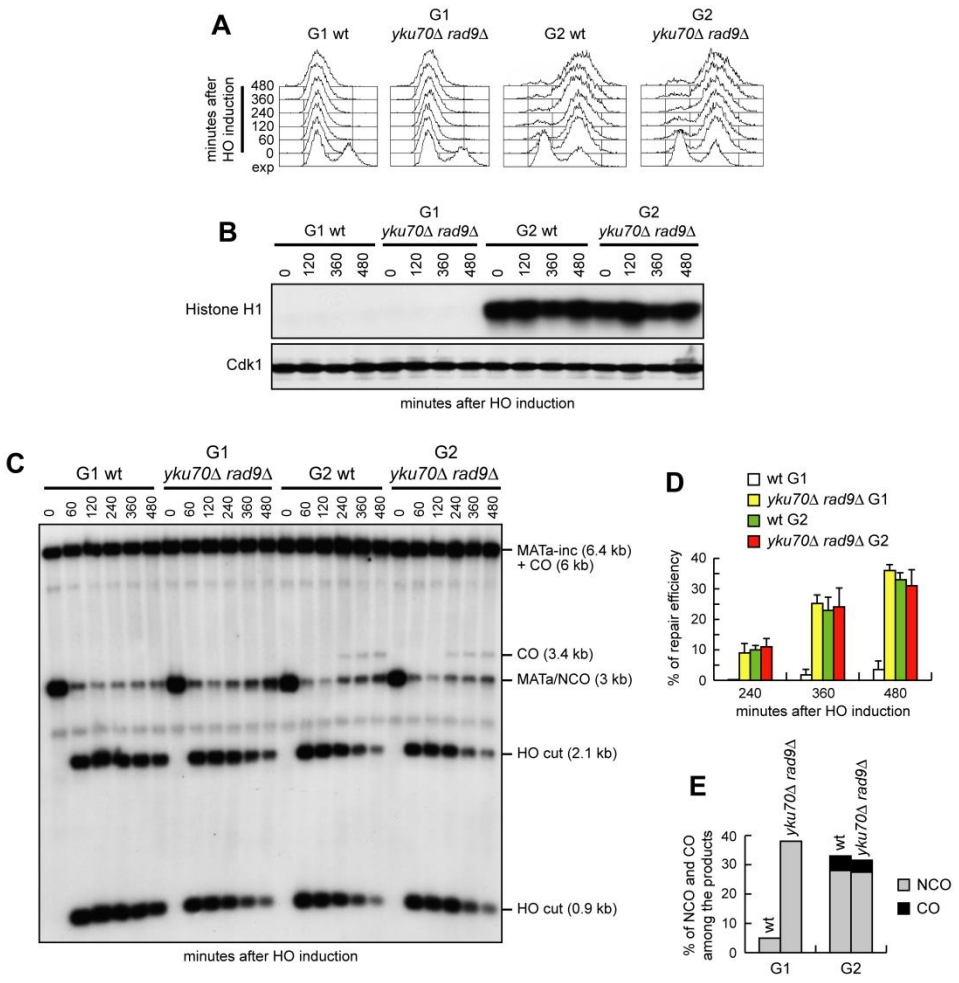

Figure 15 – **Generation of ssDNA does not bypass Cdk1 requirement for crossover recombination.** Exponentially growing YEP+raf (exp) wild type and *yku70Δ rad9Δ* cells carrying the system described in Figure 14A were arrested at time zero in G1 with α-factor or in G2 with nocodazole, and transferred to YEP+raf+gal in the presence of α-factor or nocodazole, respectively. (A) FACS analysis of DNA content. (B) Cell samples of strains expressing fully functional Cdc28-HA were taken at the indicated times to assay Cdk1 kinase activity (top row) and to determine Cdk1 levels (bottom row) as in Figure 9F. (C) Southern blot analysis of EcoRI-digested genomic DNA as described in Figure 14. (D) Densitometric analysis of repair band signals (CO+NCO). Plotted values are the mean value ±SD from four independent experiments as in (C), enclosing that described in (B). (E) Densitometric analysis of CO versus NCO repair bands at 480 minutes from break induction. See Materials and Methods for details.

If the inability to perform crossover recombination in G1 were due to the lack of Cdk1 activation, then ectopic expression of active Cdk1 should allow crossover recombination in G1, whereas Cdk1 inhibition should prevent crossover formation in G2. We then constructed wild type and *yku70Δ rad9Δ* strains carrying the system in Figure 14A and expressing a stable version of the mitotic cyclin *CLB2* under the control of the *GAL* promoter (*GAL-CLB2dbΔ*). This Clb2 variant forms active Clb2-Cdk1 complexes also during G1, because it lacks the destruction box, and therefore it is not subjected to B-type cyclin-specific proteolysis (Amon *et al.*, 1994). Strikingly, when both DSB formation and Clb2db*Δ* overproduction were induced in G1-arrested cell cultures by galactose addition (Figure 16A), crossover products became detectable in both *GAL-CLB2dbΔ* and *yku70Δ rad9Δ GAL-CLB2dbΔ* cells, whereas they were not present in wild type and *yku70Δ rad9Δ* cells under the same conditions (Figure 16B and 16C).

To assess whether Cdk1 inhibition prevented crossover formation in G2, we compared the products of interchromosomal recombination in G2-arrested *yku70Δ rad9Δ* and *yku70Δ rad9Δ GAL-SIC1ntΔ* cells (Figure 16D), the latter expressing high levels of a stable version of the Cdk1 inhibitor Sic1 (Sic1nt*Δ*) (Desdouets *et al.*, 1998). When both DSB formation and Sic1nt*Δ* overproduction were induced in G2-arrested cell cultures by galactose addition, crossover products accumulated, as expected, in *yku70Δ rad9Δ* cells, but they were undetectable in *yku70Δ rad9Δ GAL-SIC1ntΔ* cells (Figure 16E and 16F).

Thus, Sic1-mediated Cdk1 inhibition prevents generation of crossover products in G2, whereas ectopic Cdk1 activation leads to crossover recombination in G1, supporting the hypothesis that Cdk1 activity is required to promote crossover HR events even when DSB resection is allowed by the absence of Yku and Rad9.

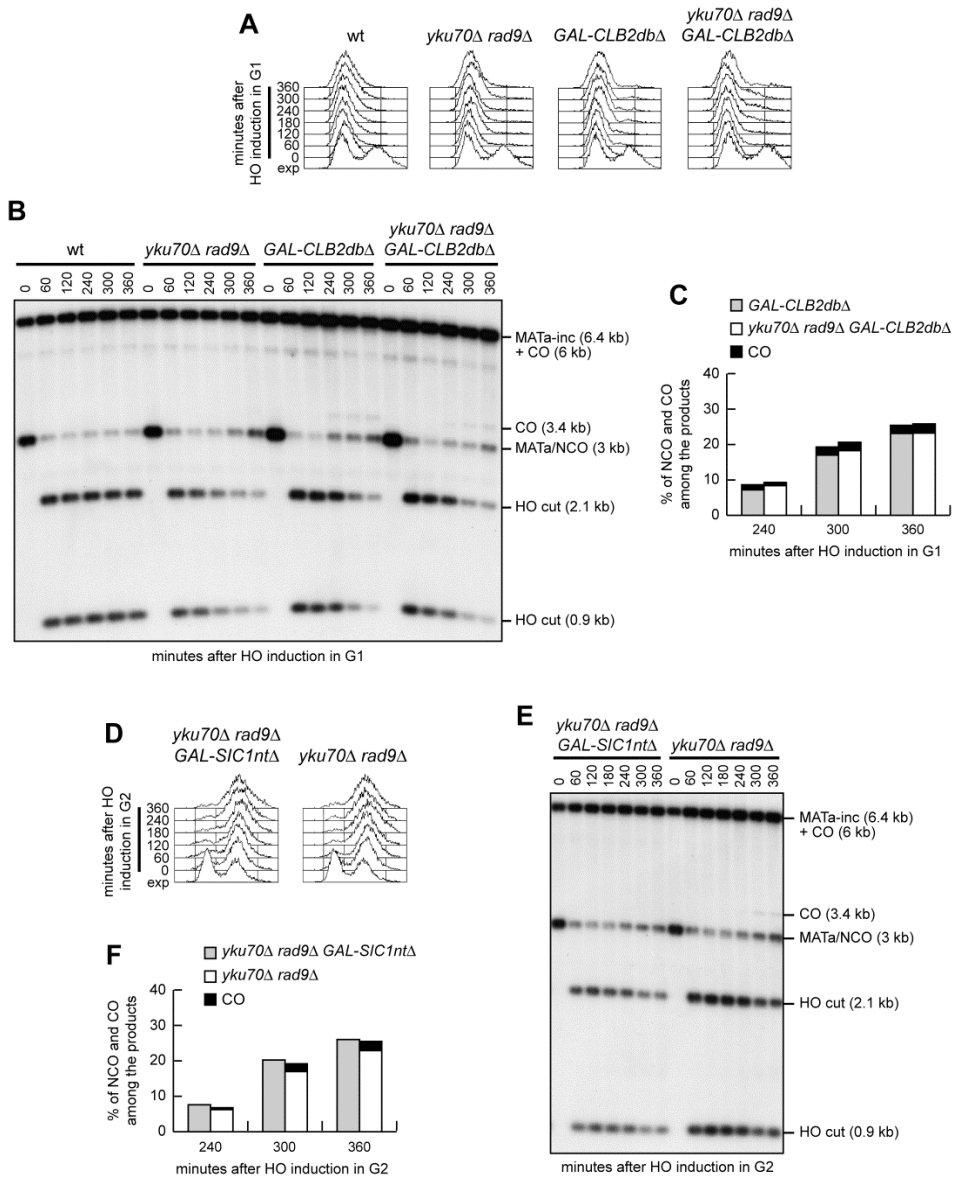

Figure 16 – **Ectopic Cdk1 activation allows crossovers in G1, whereas Cdk1 inhibition prevents crossover in G2.** (A-C) Exponentially growing YEP+raf (exp) cultures of cells with the indicated genotypes and carrying the system described in Figure 14A were arrested at time zero in G1 with α-factor and transferred to YEP+raf+gal in the presence of α-factor. (A) FACS

analysis of DNA content. (B) Southern blot analysis of EcoRI-digested genomic DNA as described in Figure 10. (C) Densitometric analysis. Plotted values are the mean value from two independent experiments as in (B). (D-F) Exponentially growing YEP+raf cells with the indicated genotypes and carrying the system described in Figure 14A were arrested at time zero in G2 with nocodazole and transferred to YEP+raf+gal in the presence of nocodazole. (D) FACS analysis of DNA content. (E) Southern blot analysis of EcoRI-digested genomic DNA as described in Figure 14. (F) Densitometric analysis. Plotted values are the mean value from three independent experiments as in (E).

Interplay between yeast Thioredoxin Reductase and Recombination Pathways in response to Replication Stress

Genetic screening for extragenic suppressors of *mre11Δ* HU sensitivity.

The MRX (Mre11-Rad50-Xrs2) complex is involved in several aspects of the DNA metabolism, such as double strand breaks (DSB) recognition and repair, meiotic recombination and telomeres homeostasis (Myung et al., 2001). Moreover, deletion of each component of the MRX complex causes phenotypes which are likely associated to DNA replication defects, such as high rates of gross chromosomal rearrangement and sensitivity to the replication inhibitor hydroxyurea (HU) (D'Amours and Jackson, 2002). HU inhibits the ribonucleotide reductase enzyme (Rnr1), thus depleting the pool of dNTPs and causing the block or the slowing down of replication fork progression. It has been recently proposed that the MRX complex participates in maintaining the replisome associated to the DNA in the presence of replication stress independently from the S-phase checkpoint (Tittel-Elmer et al., 2009). In particular has been proposed that MRX complex promotes replisome stabilization during replication stress trough its tethering activity and cohesin recruitment at replication sites (Tittel-Elmer et al., 2009, 2012). However how MRX assists DNA replication and which factors cooperate with MRX during DNA synthesis is still poorly understood.

In order to better understand why cells lacking a functional MRX complex die in the presence of a replication stress, we performed a genetic screening searching for extragenic suppressors of *mre11Δ* HU sensitivity. Briefly, as *mre11Δ* cells do not form colonies on plates containing 40mM HU (Figure 17A), we plated almost 1.200.000 *mre11Δ* cells, from 60 independent clones, on YEPD plates containing 40mM HU, searching for clones able to form colonies. More than hundred clones able to grow on HU containing plates were identified. Among them, 27 were confirmed as good suppressors of HU sensitivity. We performed dominance test in order to distinguish between recessive, dominant, and semi-dominant mutations crossing each mutant

strain with the original *mre11Δ* strain to analyze the HU sensitivity of the diploid obtained. We discovered that 12 mutants carried recessive mutations and we focused on these suppressors in order to understand if the HU resistance was due to mutations in a single gene. By crossing these recessive clones with a *MRE11* strain, we found that the suppressor phenotype for two of them was due to a single-gene recessive mutation. Furthermore, a complementation test showed that these two clones, that we named *sms1-2* and *sms1-6* (suppressor of mre11 sensitivity 1), carried mutations in the same gene. *sms1-2* and *sms1-6* mutants showed slight growth defects on YEPD and sensitivity at 37°C, however these alleles were able to partially suppress the HU sensitivity of *mre11Δ* cells as the double mutants *mre11Δ sms1-2* and *mre11Δ sms1-6* formed colony more efficiently than *mre11Δ* cells in presence of HU (Figure 17A).

Mutations in *TRR1* gene suppress the HU sensitivity of *mre11Δ* cells.

Both the clones identified showed growth defects at 37°C (Figure 1A). Tetrads analysis obtained by crossing the identified *sms1* mutants with a *MRE11* strain showed that the HU resistance phenotype co-segregates with the temperature sensitivity at 37°C, indicating that the inactivation of *SMS1* gene causes the temperature sensitivity and that the *SMS1* gene is essential to support viability at high temperature. As this phenotype was recessive, we decided to clone the *SMS1* gene by searching in a yeast genomic library plasmids that complement the temperature-sensitive phenotype of the *sms1* mutants.

We used a yeast genomic DNA library constructed in the pUN100 centromeric vector to transform both *sms1-2* and *sms1-6* mutants. We plated these cells at 37°C in order to recover the plasmids from colonies which were able to grow at this temperature. We collected three plasmids from *sms1-2* transformants and two plasmids from *sms1-*

6 transformants and after restriction analysis with EcoRI enzyme we decided to sequence the inserts from three of these plasmids. The only gene in common between all genomic inserts were *TRR1*, encoding for thioredoxin reductase enzyme. To further confirm that the HU sensitivity suppression phenotype was due to the thioredoxin reductase, we constructed a new plasmid subcloning *TRR1* gene in a *LEU2* centromeric vector with EcoRI enzymatic digestion. This plasmid was used to transform the original *sms1-2* mutant demonstrating that *TRR1* was able to suppress the temperature sensitivity phenotype typical of *sms1-2* cells. this result indicated that *sms1-2* mutant was mutated in the *TRR1* gene. Furthermore the suppression of *mre11Δ* HU-sensitivity was complemented by the same plasmid (Figure 1B).

To test if loss of Trr1 function was responsible for the suppression of the *mre11Δ* HU sensitivity by *trr1-2* and *trr1-6*, we deleted *TRR1* gene in *mre11Δ* cells. Although *mre11Δ trr1Δ* double mutant formed colonies less efficiently than *mre11Δ* cells in the absence of HU, *mre11Δ trr1Δ* cells formed colonies more efficiently than *mre11Δ* cells in HU containing plates, indicating that Trr1 inactivation improves the viability of *mre11Δ* cells in the presence of HU (Figure 17C). Thus we decided to call these mutants as *trr1-2* and *trr1-6*.

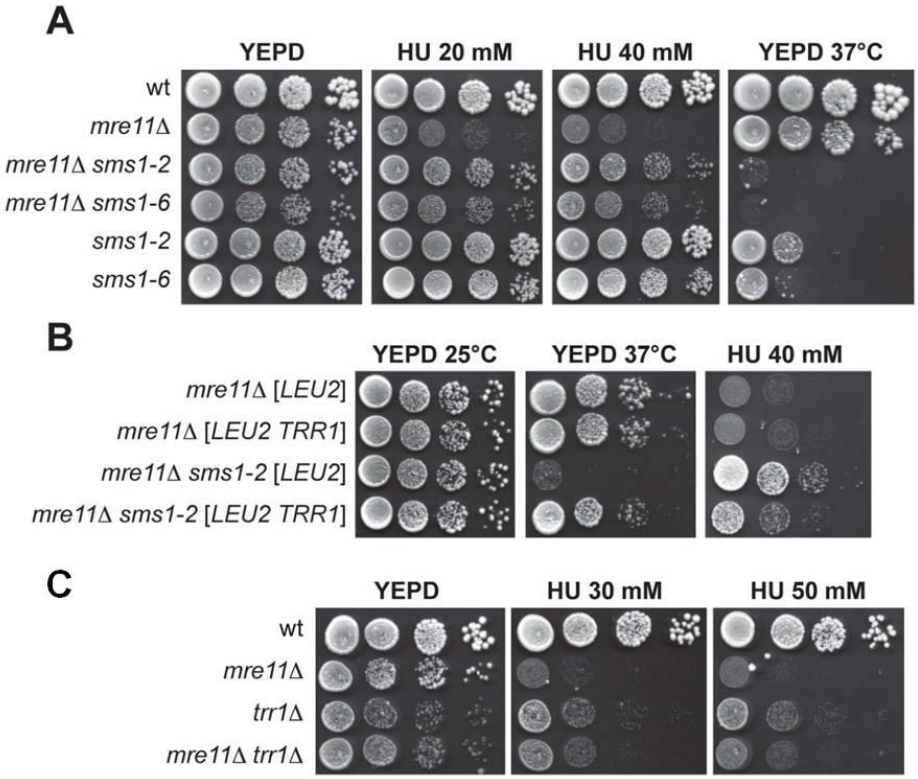

Figure 17 - **Mutation in *TRR1* gene partially suppresses the HU sensitivity of *mre11Δ* cells** - Exponentially growing cultures of strains with the indicated genotypes were serially diluted (1:10), and each dilution was spotted out on YEPD plates with or without HU at the indicated concentrations. Plates were incubated at 25°C for 3 days or, where indicated, at 37°C for 2 days.

Loss of Trr1 function improves viability of *mre11Δ* mutants under replication stress.

TRR1 encodes for the cytoplasmic thioredoxin reductase, which is required for the thioredoxin antioxidant pathway and the resistance to oxidative stress (Chae et al., 1994; Meyer et al., 2009; Morano et al., 2012). We found that, similarly to *TRR1* deletion (Trotter and Grant, 2005), *trr1-2* and *trr1-6* alleles conferred sensitivity to both high temperature and treatment with hydrogen peroxide, although less severe than those caused by *TRR1* deletion (Figure 18A). Furthermore, while *trr1Δ* cells exhibited a strong slow growth phenotype also in reach medium at 25°C, *trr1-2* and *trr1-6* cells showed and intermediate phenotype between wild type and *trr1Δ* cells in these conditions (Figure 18A). These results indicate that *trr1-2* and *trr1-6* are partial loss of function alleles of *TRR1* gene, and that *trr1-2* and *trr1-6* mutations compromised the antioxidant function of Trr1.

As *trr1-2* and *trr1*-6 alleles have similar effects of the lack of Trr1, the *trr1-2* and *trr1-6* mutations could affect Trr1 protein synthesis or stability. We therefore constructed strains where the chromosomal wild type *TRR1*, *trr1-2* or *trr1-6* coding regions were fused with Myc epitopes. Western blot analysis with anti-Myc antibodies revealed similar amounts of Trr1-Myc, Trr1-2-Myc and Trr1-6-Myc in *TRR1-MYC*, *trr1-2-MYC* and *trr1-6-MYC* protein extracts (Figure 18B).

Altogether, these results indicate that *trr1-2* and *trr1-6* are partial loss of function alleles of thioredoxin reductase. Thus, loss of Trr1 function partially restores viability of cells lacking Mre11 under replication stress conditions. As *trr1-2* showed the strongest *mre11Δ* HU sensitivity suppression (Figure 17A), this allele was chosen for further characterization.

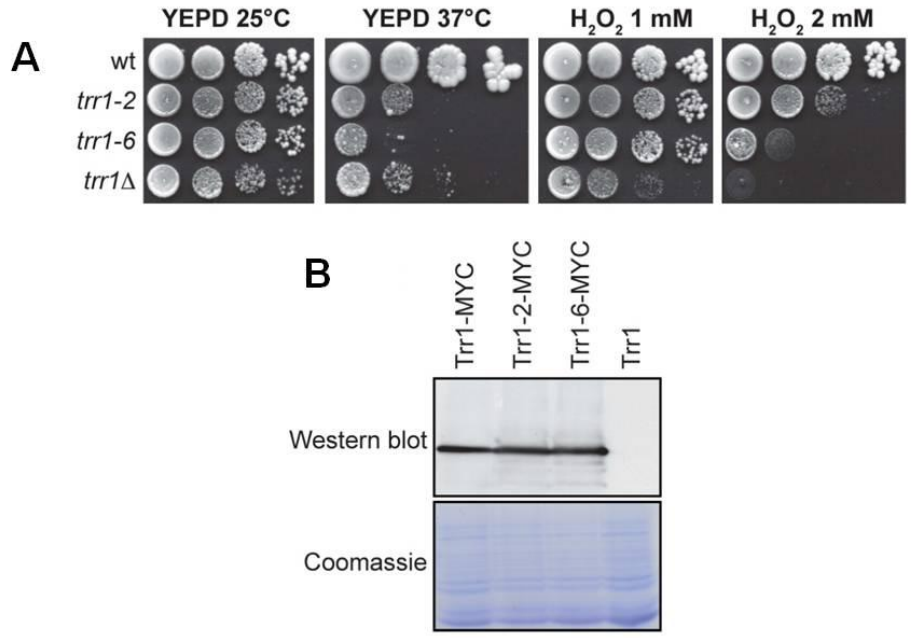

Figure 18 - ***trr1-2* and *trr1-6* are partial loss-of-function alleles of *TRR1* gene** - (A) Exponentially growing cultures of strains with the indicated genotypes were serially diluted (1:10), and each dilution was spotted out on YEPD plates with or without HU at the indicated concentrations. Plates were then incubated at 25°C for 3 days or, where indicated, at 37°C for 2 days. (B) Western blot analysis with anti-Myc antibody of protein extract from exponentially growing cells cultures expressing MYC-tagged Trr1, Trr1-2 and Trr1-6 proteins. Comassie staining was used as loading control.

trr1-2 and *trr1-6* bring single point mutation in the FAD binding domain of thioredoxin reductase.

Trr1 is a member of the pyridine nucleotide-disulfide oxidoreductase family of the flavoenzymes, which use a dithiol-disulfide active-site to transfer reducing equivalents from NADPH to thioredoxin, via the cofactor FAD (Lennon et al., 2000). The sequencing of the wild type and mutant *TRR1* coding regions revealed that both the *trr1-2* and *trr1-6* allele carried a single base pair substitution causing the amino acid substitution A18D and I116S in *trr1-2* and *trr1-6* respectively (Figure 19A). Both these

substitutions are in the Trr1 FAD binding domain (Figure 19A) and involve residues that are highly conserved in thioredoxin reductases from different organisms (Figure 19B; Oliveira et al., 2010; Zhang et al., 2009). As the FAD binding is important for the enzymatic activities of thioredoxin reductases, as to catalyze the disulfide reduction of oxidized thioredoxins (Lennon et al., 2000), one possibility is that the aminoacid substitutions of Trr1-2 and Trr1-6 protein variants are defective in their enzymatic functions.

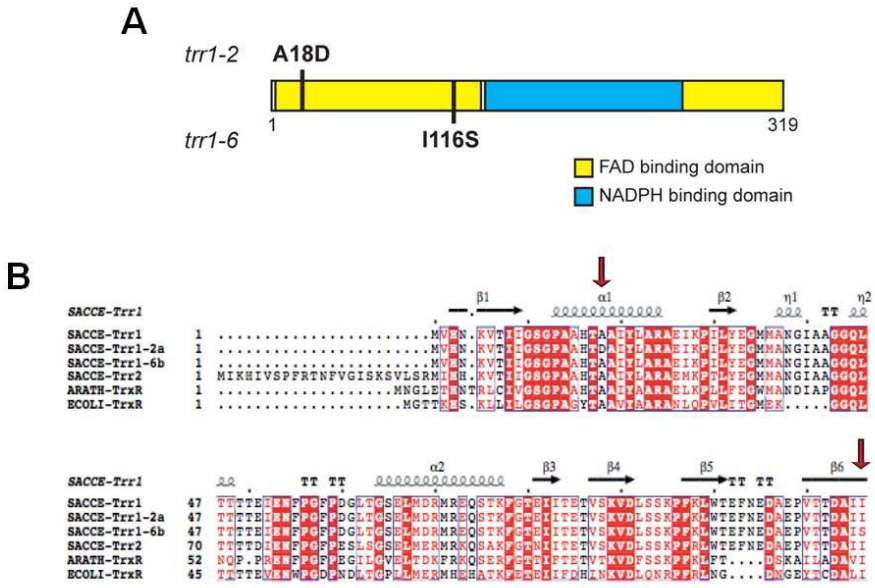

Figure 19 – ***trr1-2* and *trr1-6* mutation affect FAD binding domanin of Trr1 protein.** (A) Schematic rappresentation of Trr1 protein. Two conserved domain are colored in yellow (FAD binding domain) and in blue (NADPH binding domain). Mutations of each mutant are indicated. (B) Allignment of protein sequence of Trr1, Trr1-2, Trr1-6, Trr2 of *S. cerevisiae*, TrxR of *A. thaliana* and *E. coli*. Residues conserved in all seqeuces are writed in white on red beckground. The indicated secondary structure is reffered to Trr1 of *S.cerevisiae*. Mutations are indicate with red arrows.

The HU sensitivity of *mre11Δ* cells is not due to a deregulation of deoxyribonucleotide (dNTPs) levels.

Perturbations in the absolute and relative concentrations of the four dNTPs increase mutation rates by reducing the fidelity of DNA synthesis (Reichard, 1988). Moreover, it has been recently demonstrated that reduction of the dNTP pool has antimutator effects and enhances DNA replication fidelity *in vivo* (Laureti et al., 2013). Changes in dNTPs concentration may occur due to mutations in enzymes involved in dNTP metabolism or changes in the environment.

Thioredoxins were identified based on their ability to serve as a substrate of reducing equivalents for ribonucleotide reductase (Laurent et al., 1964). More recently has been demonstrated that in *S. cerevisiae* Trx1 and Trx2 function as the major reductants of ribonucleotide reductase during S phase (Camier et al., 2007; Koc et al., 2006). Thus, it is not surprising that in the *trx1Δ trx2Δ* double mutant dNTPs levels are affected. However, how the double deletion of thioredoxins influence the dNTPs pool is not so obvious. Indeed, at first Muller and coworkers demonstrated that in asynchronously growing cells dNTPs pools are 40% higher in *trx1Δ trx2Δ* double mutant compare to wild type (Muller, 1991). Later studies performed on synchronously growing cultures demonstrated that thioredoxins deficient mutant fail to increase the amounts of dNTPs once entered in S-phase. Thus, while in G1 phase dNTPs levels are higher in *trx1Δ trx2Δ* cells respect to wild type cells, during DNA synthesis dNTPs are lower in *trx1Δ trx2Δ* compare to that of wild type (Koc et al., 2006).

As thioredoxin reductase functions together with Trx1 and Trx2 (Gan, 1991) we asked if a modulation of dNTPs levels during the cell cycle, due to a partial inactivation of Trr1, could be responsible for the HU sensitivity suppression of *mre11Δ* cells. To test

this hypotesis we studied if variations in the levels of dNTPs in both senses were able to influence the HU sensitivity of an *mre11Δ* strain.

At first we tested whether increasing the dNTP pool is sufficient to restore the viability of *mre11Δ* cells in HU. We induced an increase in dNTP levels by overexpressing the two ribonucleotide reductase large subunits (*RNR1* and *RNR3*) or by deleting the *SML1* in *mre11Δ*. *SML1* gene encodes for the inhibitorof the Rnr1 subunit (Chabes et al., 1999). We observed that nor *RNR* overexpression neither *SML1* deletion were able to influence the HU sensitivity of an *mre11Δ* strain indicating that the HU sensivity of *mre11Δ* cells is not caused by insufficient dNTP levels (Figure 4A). Than we asked if *trr1-2* suppresses the HU sensitivity of *mre11Δ* cells by decreasing the dNTP levels. If this was the case a downregulation of dNTPs production should be sufficient to restore the viability of *mre11Δ* cells in HU. *IXR1* encode for a transcriptional factor which positevely regulates the transcription of *RNR1* gene (Tsaponina et al., 2011). We inserted *IXR1* deletion in an HU *mre11Δ* strain demonstrating that although *ixr1Δ* was able to suppress the slow growth phenotype of *mre11Δ* cells on YEPD plates it was not able to influence the growth of *mre11Δ* in presence of HU. All together these results demonstrated that *trr1* mediated suppression is independet from variation in levels of dNTPs.

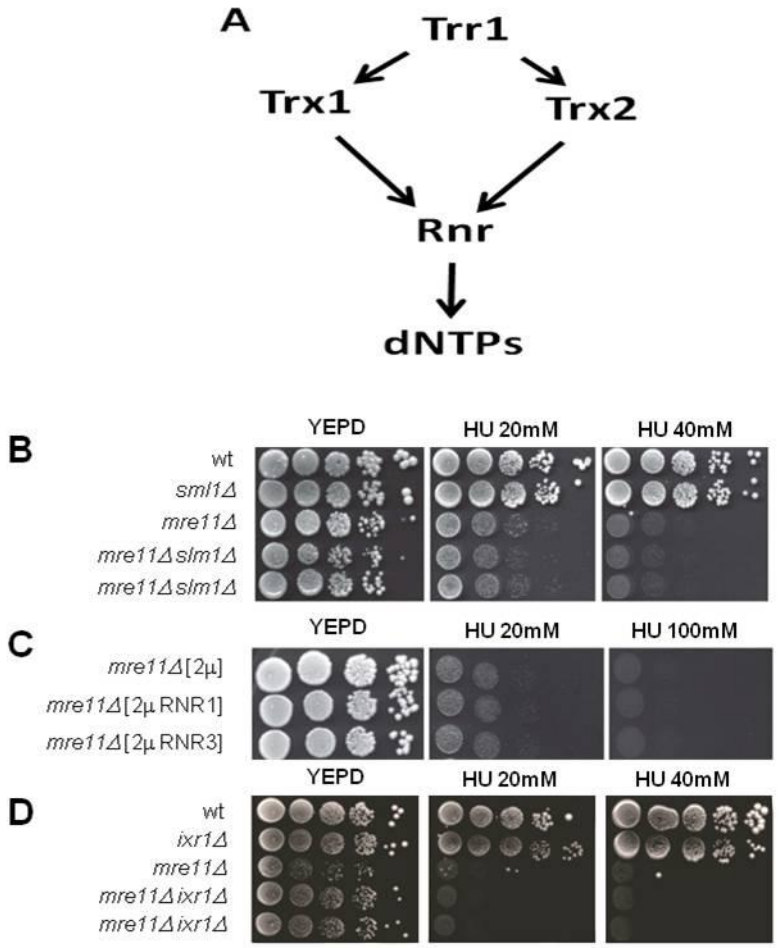

Figure 20 – **HU sensitivity of *mre11*Δ cells is independent from dNTPs –** (A) Schematic representation of interconnection between thioredxin system and dNTPs production (B-D) Exponentially growing cells cultures of strains with the indicated genotypes were serially diluted (1:10), and each dilution was spotted out on YEPD plates with or without HU at the indicated concentrations. Plates were then incubated at 25°C for 3 days. (B) Western blot analysis with anti-HA antibody of protein extract from exponentially growing cells cultures expressing HA-tagged Trx1 and Trx2 proteins. Comassie staining was used as loading control.

Thioredoxin are not required for *trr-2*-mediated suppression of *mre11Δ* HU sensitivity.

Although ribonucleotide reductase enzyme is one of the most important targets of thioredoxins systems, Trx1 and Trx2 function also on other substrates. Indeed, yeast thioredoxins act as antioxidants and play key roles in protection against oxidative stress induced by different type of ROS (Izawa et al., 1999; Kuge and Jones, 1994). Moreover most of known Trr1 functions pass through thioredoxins activities (Figure 7). The *trx1Δ* and *trx2Δ* single mutants have no obvious phenotype, but the *trx1Δ trx2Δ* double mutant is not able to grow on sulfate as sole sulfur source, and is hypersensitive to oxidants (Mouaheb et al., 1998; Muller, 1995). Although most thioredoxins functions are redundant they also act in a specif manner. Trx2 is induced by oxidative stress, interacts more efficiently with the Tsa1 and Ahp1 peroxiredoxins than Trx1, whereas Trx1 interacts strongly with the PAPS reductase (Vignols et al., 2005).

In order to understand if the activity of thiredoxin is detrimental for cells lacking the MRX complex as Trr1 inactivation is, we inserted single or double deletion of *TRX1* and *TRX2* in an *mre11Δ* strain and test the ability of the resulting strains to grow in HU.

The single deletions of *TRX1* or *TRX2* did not affect the growth of *mre11Δ* on YEPD plates and were not able to restore the viability of *mre11Δ* cells on HU containing plates (Figure 21A). While *TRR1* deletion caused slow growth phenotype on YEPD plates (Figure 17C, 21A) the double mutant *trx1Δ trx2Δ* did not cause any growth defects in the same conditions, indicating that Trr1 could has other roles in addition to thioredoxins regulation. Despite these differences the deletion of *MRE11* negatively influenced the growth of *trx1Δ trx2Δ* double mutant on YEPD plates while did not affect the growth of *trr1Δ* cells in the same conditions (Figure 21A). In

presence of HU the single deletion of *TRR1* was able to partially restore the viability of *mre11Δ* cells while the *trx1Δ trx2Δ* double mutant did not suppress the HU sensitivity caused by *MRE11* deletion (Figure 21A). Thus, we concluded that also the double deletion of thioredoxins was not able to suppress the HU sensitivity of *mre11Δ* cells.

It has been demonstrated that mutations in *TRR1* cause an increase in the expression of Trx2 (Carmel-Harel et al., 2001). Moreover, TRX2 has been found as strog induced in response to oxidative stress (Carmel-Harel et al., 2001). Thus, we asked if mutations in *TRR1* or oxidative stress condition were able to induce an increase of thioredoxins levels. As we observed that *trr1-2* suppressed *mre11Δ* HU sensitivity we asked if also this replicative stress was able to induce thioredoxin expression. We therefore constructed strains where the chromosomal wild type *TRX1*, or *TRX2* coding regions were fused with HA epitopes in order to follow how vary thioredoxins levels in *trr1-2* mutant with or without stress conditions. Protein extracts were prepared from exponentially growing cells, untreated or treated with with 40mM of HU or 3mM of H_2O_2. Western blot analysis with anti-HA antibodies revealed that variations of thioredoxins levels in response of stress consition were not detectable with this assay while an increase in the amounts of both Trx1-HA and Trx2-HA protein were visible in presence of mutation in *TRR1*, with Trx2-HA stronger induced than Trx1-HA (Figure 21B).

Starting from this data we speculated that the increase of thioerdoxins levels could be responsible for the suppression of the *mre11Δ* HU sensitivity caused by *trr1-2* allele. If this were the case, we expected the the thioredoxin overexpression increase the viability of *mre11Δ* cells on HU-containing plates. Thus, we overexpressed both thioredoxins in *mre11Δ* cells, by transforming wild type and *mre11Δ* cells with multicopy plasmids carrying *TRX1* and *TRX2* genes. Thioredoxins overexpression did not affect the viability on YEPD. Moreover, it was not able to suppress the HU

sensitivity of *mre11Δ* cells (Figure 21C). From these results we concluded that *trr1-2* suppresses the HU sensitivity of *mre11Δ* cells independently from thioredoxin, raising the hypotesis of a new role of thioredoxin reductase yet to be discovered.

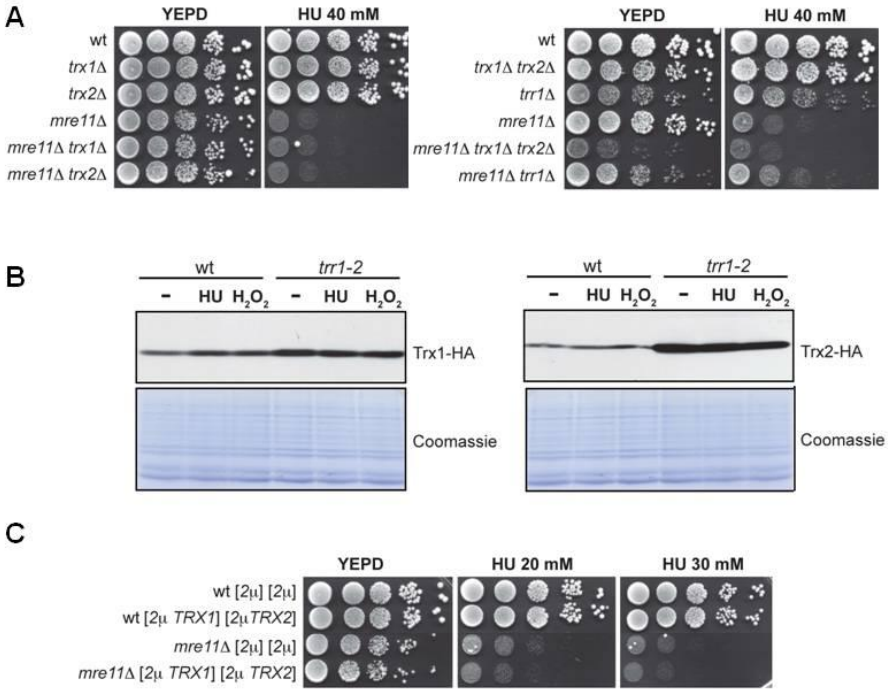

Figure 21 – **HU sensitivity of *mre11*Δ cells is independent from thioredoxins.** (A-C) Exponentially growing cells cultures of strains with the indicated genotypes were serially diluted (1:10), and each dilution was spotted out on YEPD plates with or without HU at the indicated concentrations. Plates were then incubated at 25°C for 3 days. (B) Western blot analysis with anti-HA antibody of protein extract from exponentially growing cells cultures (untreated or treated with 40mM of HU or 3mM of H_2O_2) expressing HA-tagged Trx1 and Trx2 proteins. Comassie staining was used as loading control.

Suppression of the *mre11Δ* HU sensitivity by loss of Trr1 function does not require *YAP1* transcription factor.

We demonstrated that thioredoxin were not involved in the suppression of HU sensitivity of an *mre11Δ* strain. However we observed that thioredoxins levels were higher in *trr1-2* mutant compared to wild type cells. *TRX2* gene transcription is induced by Yap1 transcription factor in response to oxidative stress, nevertheless Yap1 induce transcription of many other antioxidant genes (Carmel-Harel et al., 2001). We asked if *trr1-2*-mediated suppression of *mre11Δ* HU sensitivity could be due to an iperactivation of the global transcriptional response mediated by Yap1. Since *YAP1* deletion was found to cause a sintetic sick phenotype when combined with deletion of MRX complex subunits (Bandyopadhyay et al., 2010), we can not analyse the HU sensitivity of the triple mutant *mre11Δ trr1-2 yap1Δ.* Hence, we tested if *YAP1* overexpression was able to suppress the HU sensitivity of *mre11Δ* cells. We trasformed wild type and *mre11Δ* strains with 2μ multicopy plasmid carriyng the *YAP1* coding sequence or with empty plasmid. We observated that *YAP1* overexpression did not affect the growth of both wild type and *mre11Δ* on YEPD plates, however it is not able to suppress the HU sensitivity of *mre11Δ* (Figure 22).

These results demonstrated that nor thioredoxins overexpression neither a global transcriptional response mediated by Yap1 were responsible for the *mre11Δ* HU sensitivity suppression mediated by *trr1-2*.

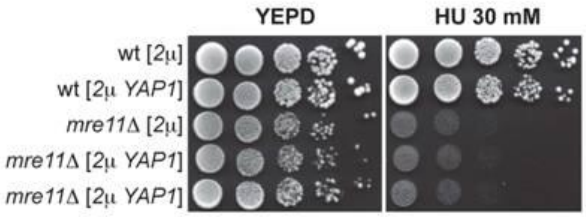

Figure 22 – **Yap1 transcription factor is not involved in *trr1*-mediated suppression of the *mre11Δ* HU sensitivity**. Exponentially growing cultures of strains with the indicated genotypes were serially diluted (1:10), and each dilution was spotted out onto YEPD plates with or without HU at the indicated concentrations. Plates were then incubated at 25°C for 3 days.

Inactivation of the thioredoxin reductase Trr1 improve viability of recombination mutants to replication stress

The S phase checkpoint and the homologous recombination (HR) pathways are the two main mechanisms required to maintain genome integrity during DNA replication by ensuring replisome stability and recovery after fork collapse (Li and Heyer, 2008; Tercero et al., 2003). Indeed several mutants in checkpoint or HR factors are sensitive to HU (D'Amours and Jackson, 2001). As MRX complex is implicated in both checkpoint activation and homologous recombination (D'Amours and Jackson, 2002) we asked if *trr1-2* allele was also able to suppress other checkpoint or HR mutants.

We combined *trr1*-2 allele with recombination mutants *rad51Δ*, *rad52Δ*, and *sae2Δ* and checkpoint mutants *mec1Δ*, and *mrc1Δ* in order to check if loss of Trr1 function was able to restore the viability of these mutants on HU-containing plates. Sae2 cooperate with MRX complex during the firts step of endonucleolitic resection of 5' DNA ends, while Rad52 is requred to load Rad51 on ssDNA. Once loaded on DNA Rad51 nucleofilemnt promote searching and invasion of homologous sequences (Longhese et al., 2010). Mec1 belongs to PI3-like kinase family and functions as the main sensor of S-phase checkpoint in budding yeast, where is recruited to single strand by RPA and signals the presence DNA damage to adaptor proteins like Mrc1 which is specific for replication checkpoint (Segurado and Tercero, 2009).

Surprisingly, in addition to mre11 (Figure 23A), *trr1-2* was able to suppress HU sensitivity of recombination mutants *rad51Δ, sae2Δ* (Figure 23B, C) and *rad52Δ* (data not shown) while it did not suppress the HU sensitivity of *mec1Δ* and mrc*1Δ* mutants checkpoint mutants(Figure 23D, E).

These results demonstrated that *trr1-2* specifically suppress the HU sensitivity of recombination mutants. Thus, Trr1 activity is deleterious during replication stress in the absence of functional recombination machinery. Instead, checkpoint mutants HU sensitivity is not suppressed by *trr1-2* allele raising the possibility that the checkpoint is involved in Trr1-2 mediated suppression.

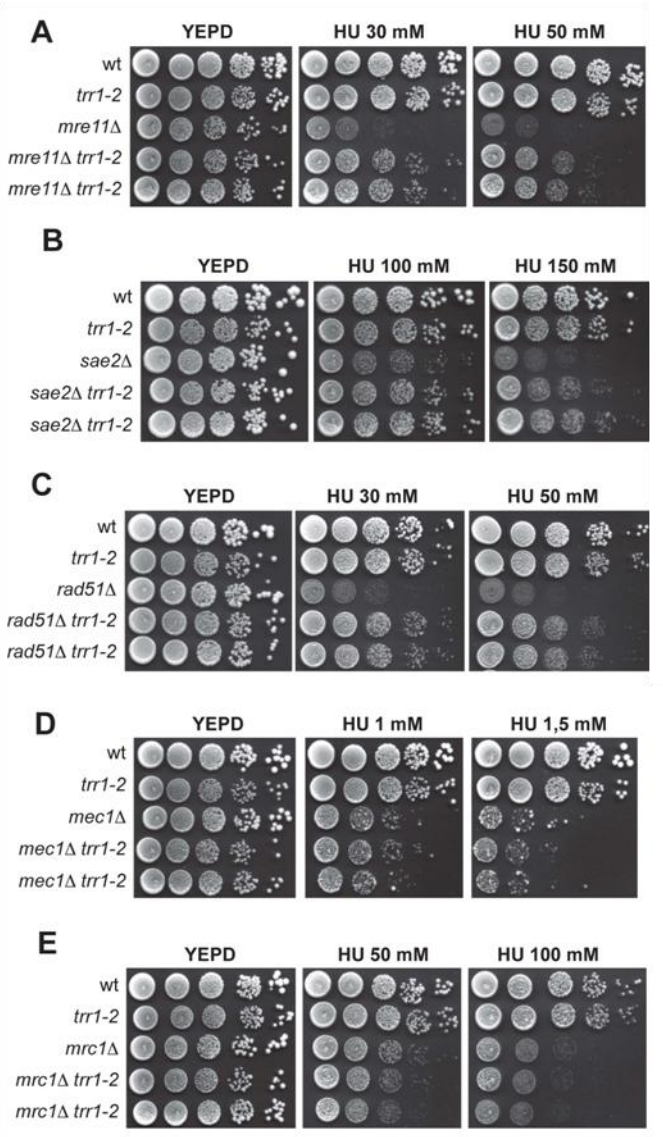

Figure 23 – **Loss of Trr1 function specifically suppress HU sensitivity of recombination mutants.** Exponentially growing cultures of strains with the indicated genotypes were serially diluted (1:10), and each dilution was spotted out on YEPD plates with or without HU at the indicated concentrations. Plates were then incubated at 25°C for 3 days.

The loss of Trr1 function improves checkpoint activation in recombination mutatnts during replication stress.

S-phase checkpoint has two main functions: it delays nuclear division and stabilizes stalled replication forks ensuring the resumption of DNA synthesis once replication stress is removed (Segurado and Tercero, 2009). It has been reported that MRX deficient cells are defective in checkpoint activation (D'Amours and Jackson, 2001), while it is not known if also Rad51 is implicated in a proper checkpoint activation. As *trr1-2* suppresses the HU sensitivity of recombination mutants but is incapable to suppress the HU sensitivity of checkpoint mutants, we hypothesized that the HU sensitivity of recombination mutants could be due to a defect in checkpoint activation. Thus we postulated that Trr1 inactivation could suppress the HU sensitivity of recombination mutants by inducing an hyperactivation of the intra S-phase checkpoint.

In order to study the kinetics of checkpoint activation in recombination mutants under replication stress we performed an experiment to follow Rad53 phosphorylation during HU treatment. Rad53 is the main effector kinase which is phosphorilated and activated during HU treatment (Sanchez et al., 1996). Mutants in each subunit of MRX complex show a defect in Rad53 activation (D'Amours and Jackson, 2001) while nobody have demonstrated the involvment of Rad51 in checkpoint activation.

We tested if *trr1-2* allele was able to restore Rad53 phosphorilation in *mre11Δ* and *rad51Δ* mutants. *mre11Δ trr1-2* and *rad51Δ trr1-2* cells and their control strains were

bocked in α-factor and released in fresh medium with 40 mM of HU. Rad53 phosphorylation was followed by western blot with Rad53 specific antibody.

Rad53 phosphorylation is visible on western blot as the appearance of upper bands. In wild type cells released in HU, Rad53 phosphorylated forms appeared at 30 minutes and was maintain until 150 minutes (Figure 24). In *trr1-2* mutant Rad53 phosphorylation followed the same kinetics of the wild type in terms of time, however phosphorylation was stroger induced respect to wild type as demonstrated by the differences in ratio between upper and lower bands intensity (particularly at 60, Figure 24). *mre11Δ* cells showed defects in Rad53 phosphorylation as the upper band never appeared. Surprisingly also *rad51Δ* cells showed a defect in Rad53 activation since the upper band which was well defined in wild type was not clearly visible in *rad51Δ* cells, however, this defect is less if compare to that of *mre11Δ* cells. *trr1-2* allele was able to partially restore Rad53 phosphorilation in both *mre11* and *rad51* mutant strains (Figure 24). In fact, the upper band which was not clearly visible in both *mre11Δ* and *rad51Δ* cells returned distinguishable in both *mre11Δ trr1-2* and *rad51Δ trr1-2* double mutants (Figure 24).

All together these results demonstrated that *trr1-2* allele alone influnces the Rad53 phosphporylation in response to HU treatment enhancing the amount of Rad53 phosphorylated form. Moreover, *trr1-2* allele in combination with *MRE11* or *RAD51* deletions is able to suppress the Rad53 phosphporylation defects typical of these mutants. If this Rad53 reactivation depends on a direct action of Trr1 on checkpoint pathway or it is due to an indirect effect remain to be discovered.

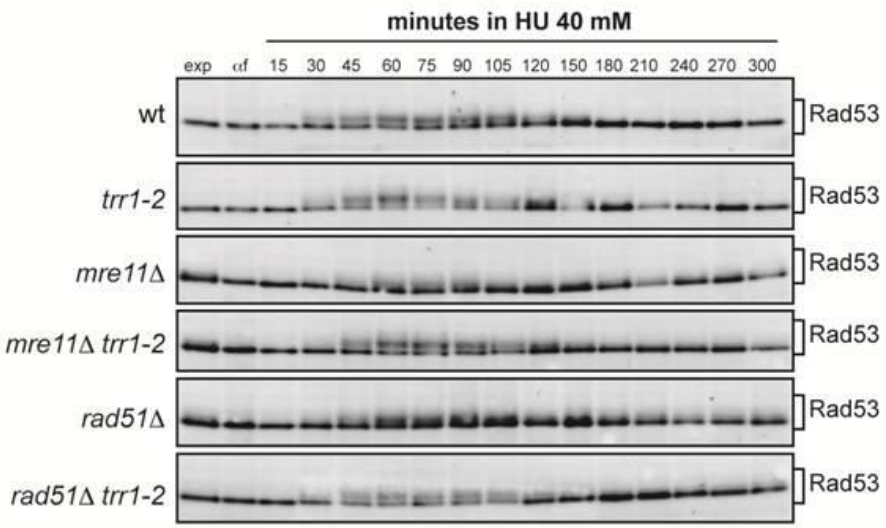

Figure 24 – ***trr1-2*** **partially restore Rad53 activation in recombination mutants.** Exponentially growing cultures of strains with the indicated genotypes were arrested in G1 with α-factor and then released from the pheromone in YEPD containing 40 mM of HU. Rad53 activation is followed by western blot analysis with anti-Rad53 specifyc antibody.

trr1-2**-mediated suppression of the recombinatuìion mutant HU sensitivity is independent of S-phase checkpoint.**

As explain above the S-phase checkpoint is important durung replication stress to delay nuclear division and stabilize replication forks ensuring the resumption of DNA synthesis once replication stress is overcomed (Segurado and Tercero, 2009). The central players of S-phase checkpoint in the budding yeast *S. cerevisiae* are two kinases, namely Mec1 and Rad53 (Paulovich and Hartwell, 1995; Zhou and Elledge, 2000). Mec1 phosphorylates Mrc1 (the homologue of human Claspin), a mediator that transduces the signal from Mec1 to the effector kinase Rad53 (Alcasabas et al., 2001), which is then phosphorylated and activated (Figure 5).

We demonstrated that *trr1-2* allele does not suppress the HU sensitivity of checkpoint mutants (Figure 23C, D). Moreover we found that this mutation restores Rad53

phosphorilation which is defective in recombination mutants during HU treatment (Figure 24). These results led us to hypothesize that mutations in *TRR1* suppressed the HU sensitivity of recombination mutants improving the activation of S-phase checkpoint.

To test this possibility we combined the deletion of *MEC1* or *MRC1* checkpoint genes with the double mutant *rad51Δ trr1-2*. We chosed to use *rad51Δ* strain instead of *mre11Δ* because disruption of MRX complex cause more growth defect in combination with checkpoint mutants respect to *RAD51* deletion. We observed that checkpoint gene deletions did not affect the growth of *rad51Δ* cells in unstressed conditions (Figure 25). Conversely, on HU containing plates both *MRC1* and *MEC1* deletions decreased the viability of *rad51Δ* cells. However *trr1-2* was still able to partially suppress the HU sensitivity of both *rad51Δ mrc1Δ* and *rad51Δ mec1Δ* double mutants (Figure 25). These results demonstrated that loss of function of Trr1 could suppress the HU sensitivity of recombination mutants also in the absence of the sensor kinase Mec1 or the adaptor protein Mrc1. Thus, Trr1-2 mediated checkpoint reactivation of recombination mutants under stress conditions is only a side effect which is not responsible for thioredoxin reductase mediated suppression.

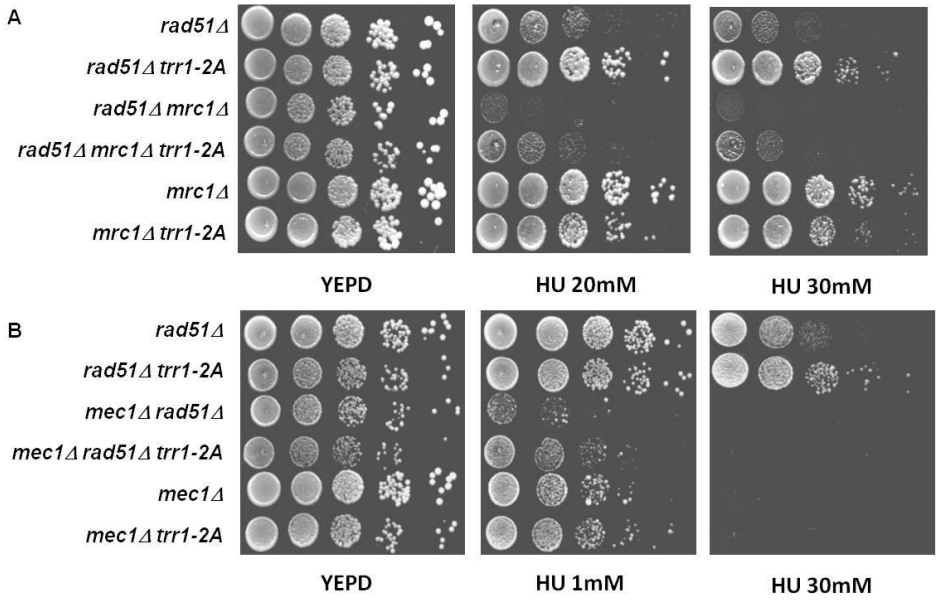

Figure 25 – ***trr1-2*** **mediated suppression of the recombination mutants HU sensitivity is independent of the S-phase checkpoint.** Exponentially growing cultures of strains with the indicated genotypes were serially diluted (1:10), and each dilution was spotted out on YEPD plates with or without HU at the indicated concentrations. Plates were then incubated at 25°C for 3 days.

trr1-2 **allele positively influences nuclear division in recombination mutants after replication stress.**

During HU treatment MRX complex is recruited at replication forks where stabilizes replisome components independently from its nuclease activity or S-phase checkpoint (Tittel-Elmer et al., 2009). According to these data, MRX deficient cells are caracterized by an instable genome with an high rate of chromosome rearrangements (Myung et al., 2001). Previous works in yeast and vertebrate demonstrated that Rad51 assists continuous DNA synthesis by preventing degradation of nascent strands at stalled forks suggesting a direct role of HR factors at stalled forks (Lambert et al., 2007; Petermann and Helleday, 2010; Petermann et al., 2010).

In order to understand if Trr1 activities hamper the resumprion of replication in recombination mutants after replication stress we studied if *trr1-2* mutation influences the recovery of DNA replication of *mre11Δ* and *rad51Δ* cells after after block of the replication forks with HU. We blocked *mre11Δ trr1-2, rad51Δ trr1-2* cells and their control strains in 200mM HU to stop DNA replication, and we released them in fresh medium without HU to permit the resumption of DNA synthesis. We then followed the kinetics of DNA replication by FACS analysis of DNA content and nuclear division by fluorescence mycroscopy. After release from the HU block, wild type cells were able to resume DNA replication reaching 2C DNA content (60 minutes, Figure 26A), divide nuclei (90 minutes, Figure 26B), and then reaccumulate DNA with 1C peak (135 minutes, Figure 26A). *trr1-2* cells showed a DNA replication and nuclear division kinetics similar to wild type cells. Also *mre11Δ* cells were able to resume DNA replication reaching 2C DNA content (60 minutes, Figure 26A) and, most of them showed a defect in reaccumulation of 1C DNA peak and nuclear division (Figure 26A, B). It has been demonstrated that *mre11Δ* cells accumulated stretch of unreplicated DNA during treatment with HU (Tittel-Elmer et al., 2009). Thus, we postulated that defects in nuclear division were due to failure in the completion of DNA replication which hinders chromosomes segregation. *trr1-2* allele partially restore the ability of *mre11Δ* cells to reaccumulate with 1C DNA peak and divide nucleai (Figure 26 A, B). Like *mre11Δ* cells, *rad51Δ* cells were able to resume DNA replication reaching 2C DNA content but failed to return with 1C DNA peak (Figure 26A) and to divide nuclei (Figure 26B).

Reasoning on these results we speculate that homologous recombination is important to prevent the accumlation of ureplicated DNA stretch which hamper chromosome segregation and nuclear division. Trr1 loss of function could prevents the

accumulation of these unreplicated stretch promoting pathway alternative to HR or preventing the formation of structures which become subtrates of HR.

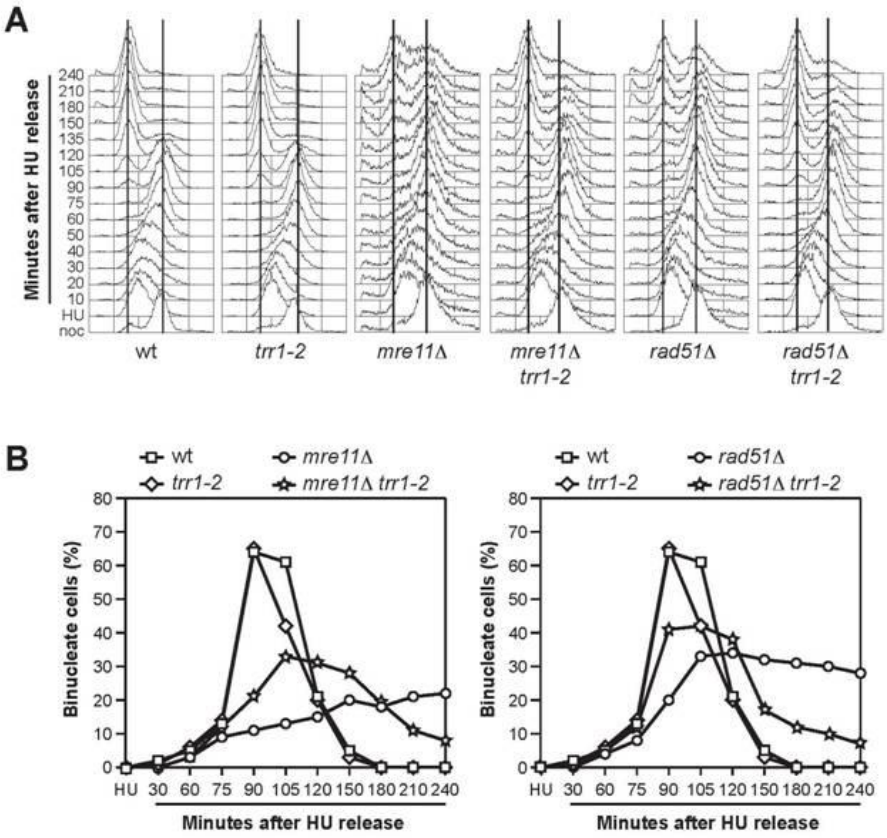

Figure 26 – **DNA replication and nuclear divsion kinetics of *mre11Δ* cells with or without mutation in *TRR1*.** Exponentially growing cells (exp) with the indicated genotypes were arrested with 200mM of HU and releaed from the block in YEPD. Aliquots of each culture were harvested at the indicated times (min.) after release from HU to determine (A) DNA content by fluorescence activated cell sorting (FACS) analysis and (B) nuclear division measured by fluorescence microscopy.

***trr1-2* mutation prevents Rad52 foci accumulation in *rad51Δ* cells exposed to replication stress.**

Agents that stall or collapse replication forks, as hydroxyurea, thymidine and camptothecin strongly induce DSB formation and homologous recombination, which promotes the survival in the presence of these treatments (Arnaudeau et al., 2001; Lundin et al., 2002). Thus, defects in HR results in the increase of unrepaired DNA lesions, among which the most deleterious double strand breaks (DSB), that are associated to genomic instability and cell death (Lisby et al., 2003).

One possibility is that *trr1-2* allele partially restores the viability of recombination mutants by preventig formation of DSB and/or increasing the frequency of double strand breaks (DSB) repair by an HR alternative mechanisms. In order to test this hypothesis we followed the DSB formation before and after HU treatment in *rad51Δ trr1-2* cells and in its control strains. It has been demonstrated that in response to DNA damage, Rad52 redistributes itself and forms foci specifically during S phase. Moreover, Rad52 foci are centers of DNA repair where multiple DNA double-strand breaks colocalize (Lisby et al., 2001, 2003). Thus we followed the DSB formation and acumulation by checking the presence of Rad52 foci in cells treated with HU. We inserted the *RAD52-YFP* construct in strains of our interest in order to monitor DSBs formation through fluorescence microscopy (Figure 27A). Exponentially growing cells were arrested in G1 phase of cell cycle with α-factor, and released in fresh medium with 40mM HU. Wild type cells did not accumulated Rad52 foci during treatment with low doses of HU and *trr1-2* allele alone did not affect foci formation (Figure 27B). About 20% of *rad51Δ* cells accumulated Rad52 foci before treatment with HU and this percentage reached 60% three hours after the addiction of HU. Only 10% of *rad51Δ trr1-2* double mutant showed foci before HU treatment and also after the HU addiction the percentage of cells with foci did not overcome 30%.

Thus, the result that *trr1-2* allele partilly suppresses foci formation in *rad51Δ* cells during HU sensitivity is in agreement with the fact that also the suppression of recombination mutants HU sensitivity is not total. These data could means that Trr1 activity promote the formation of DNA lesions or DNA structures which become substrates of recombination pathway. Thus, cells defective in recombination machinery are not able to repair these lesions and undergo to death.

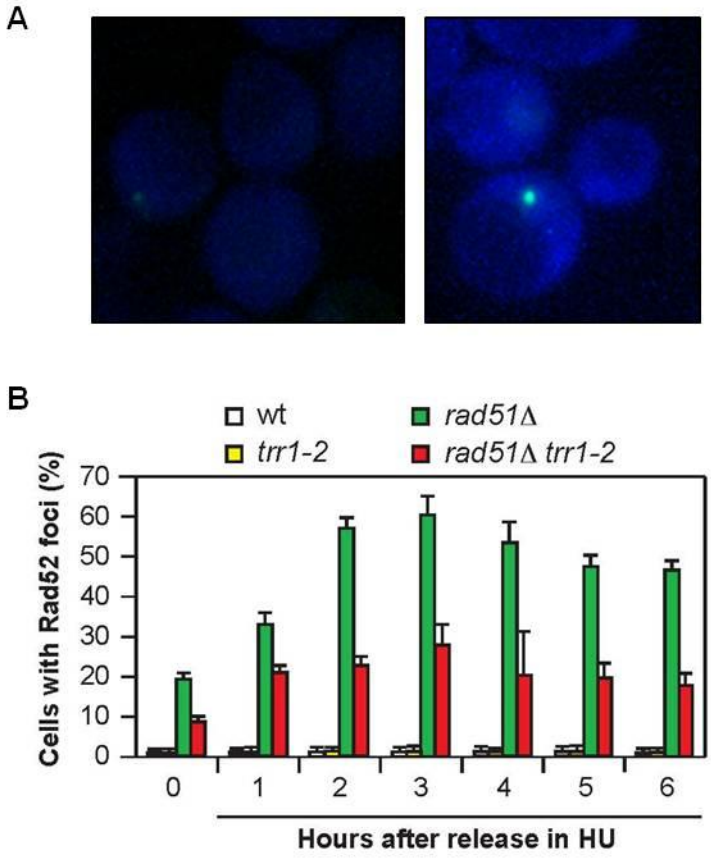

Figure 27 – ***trr1-2* allele prevents DSBs formation and acumulation before and during replication stress.** (A) Flurescence microscope images showing *rad51Δ* cell with (on the right) or without (on the left) Rad52-YFP foci. (B) Exponentially growing cells (exp) with the indicated genotypes were arrested with α-factor and released from the pheromone in YEPD containing

40mM HU. Aliquots of each culture were harvested at the indicated times (hours.) after release from HU to determine the foci formation by fluorescence microscopy.

DISCUSSIONS

Distinct Cdk 1 requirements during Single-Strand Annealing, Noncrossover, and Crossover Recombination

HR is highly coordinated with the cell cycle: it takes place predominantly during the S and G2 phases, when the presence of a sister chromatid provides a donor template and high Cdk1 activity promotes DSB end resection to expose ssDNA that is necessary to initiate HR (Aylon et al., 2004; Caspari et al., 2002; Huertas et al., 2008; Ira et al., 2004). To study whether Cdk1 plays additional role(s) in HR, we asked whether generation of ssDNA at the DSB ends is sufficient to bypass Cdk1 requirement for HR. Because the lack of either Yku or Rad9 allows Cdk1-independent generation of 3'-ended ssDNA at DSB ends (Clerici *et al.*, 2008; Lazzaro *et al.*, 2008), we investigated whether cells lacking Yku and/or Rad9 could repair a DSB by HR when Cdk1 activity is low. We found that DSB repair by SSA can take place in G1-arrested *yku70Δ* cells. The ability of these cells to carry out SSA in G1 depends on Cdk1-mediated generation of 3'-ended ssDNA at the DSB ends. In fact, the lack of Rad9 increases efficiency of both resection and SSA in *yku70Δ* G1 cells. Furthermore, Cdk1 inhibition prevents SSA in G2 wild type cells, but not in *yku70Δ rad9Δ* G2 cells, where DSB resection occurs independently of Cdk1. We also found that G1-arrested *yku70Δ* and *yku70Δ rad9Δ* cells can undergo interchromosomal recombination events that are not accompanied by crossovers. Thus, Cdk1 requirement for carrying out SSA and noncrossover recombination is bypassed by DSB resection, indicating that Cdk1 promotes these HR events essentially by regulating the resection step.

Rad52 is essential for both SSA and noncrossover recombination events, while only the latter require the assembly of Rad51 nucleoprotein filaments, which promote homologous pairing and strand exchange (reviewed in (Krogh and Symington, 2004; Pâques and Haber, 1999; San Filippo et al., 2008)). As the function of Cdk1 in DSB

repair by SSA and noncrossover recombination is primarily the regulation of the resection step, neither Rad51 nor Rad52 appear to require Cdk1 activity to exert their biochemical activities.

Interestingly, although *RAD9* deletion was shown to allow MRX-dependent DSB resection in G2 cells that overproduced the Cdk1 inhibitor Sic1 (Lazzaro *et al.*, 2008), the lack of Rad9 did not increase DSB resection or HR-mediated DSB repair in G1 compared to wild type cells. Thus, although Rad9 provides a barrier to resection in *yku70Δ* G1 cells, its lack is not sufficient, by itself, to escape the inhibitory effect of Yku on DSB resection in G1. This finding is consistent with previous data showing that the resection block imposed by Yku is relieved in G2 (Bonetti *et al.*, 2010; Shim *et al.*, 2010). It also indicates that Rad9 prevents DSB resection in all cell cycle phases, but its inhibitory effect in G1 becomes apparent only in the absence of Yku.

Surprisingly, we found that G1-arrested *yku70Δ rad9Δ* cells are specifically impaired in the formation of crossovers by interchromosomal recombination. Expression of an activated form of Cdk1 allows crossover recombination in both wild type and *yku70Δ rad9Δ* G1 cells, whereas inhibition of Cdk1 activity in G2-arrested *yku70Δ rad9Δ* cells prevents crossover formation without affecting noncrossover outcomes. These findings are consistent with a role of Cdk1 in promoting crossover recombination that is independent of its function in DSB resection.

How does Cdk1 promote crossover outcomes? The choice between crossover and noncrossover is tightly regulated (Martini *et al.*, 2006). Meiotic recombination results frequently in crossovers (Youds and Boulton, 2011), while DSB repair in mitotic cells is rarely associated with crossovers (∼5%) (Bzymek *et al.*, 2010). An explanation of these differences could be that specific mechanisms limit crossovers during mitotic homologous recombination. Indeed, dissociation of the D-loop intermediates gives rise to noncrossover products, and this process is promoted by the helicases Srs2 and

Mph1 (Ira et al., 2003; Prakash et al., 2009; Robert et al., 2006; Saponaro et al., 2010). Furthermore, noncrossover outcomes can arise also from the dissolution of dHJ intermediates that requires the combined activity of the BLM/Sgs1 helicase, which drives migration of the constrained dHJs, and the Top3-Rmi1 complex, which decatenates the interlinked strands between the two HJs (Ira et al., 2003; Lo et al., 2006; Wu and Hickson, 2003). One possibility is that Cdk1 promotes crossover recombination by inhibiting proteins specifically involved in limiting crossover generation (i.e. Sgs1, Top3-Rmi1, Srs2 and Mph1). A similar mechanism seems to act during meiotic recombination, where proteins required for homologous chromosome synapsis have been proposed to antagonize the anti-crossover activity of Sgs1 (Jessop *et al.*, 2006). However, none of the above anti-crossover proteins have been reported to undergo Cdk1-dependent inhibitory phosphorylation. Alternatively, as dHJ formation requires a transition from D-loop to second-end capture (Hunter and Kleckner, 2001), Cdk1 might favour this transition by promoting DNA synthesis and therefore by stabilizing the D-loop intermediates. Finally, Cdk1 might stimulate the activities of Mus81-Mms4, Slx1-Slx4, Yen1 and/or Rad1-Rad10 resolvases that generate crossover products by cleaving the dHJs (Svendsen and Harper, 2010). Consistent with this last hypothesis, the Yen1 and Mms4 resolvases appear to be phosphorylated by Cdk1 (Ubersax *et al.*, 2003) and the nuclease activity of mammalian Mus81-Mms4 and Yen1 are tightly regulated throughout the mitotic cell cycle (Matos *et al.*, 2011). Furthermore, *S. cerevisiae* Mms4 undergoes Cdk1-dependent phosphorylation, and this phosphorylation allows its activation as a nuclease (Gallo-Fernández *et al.*, 2012).

In conclusion, Cdk1 controls primarily DSB resection to allow SSA and noncrossover recombination, while crossover outcomes appear to require additional Cdk1-promoted events. As mitotic crossovers have the potential for deleterious genome

rearrangements, their Cdk1-dependent regulation can provide an additional safety mechanism, ensuring that the rare mitotic recombination events accompanied by crossing over at least occur in S/G2, when a sister chromatid is available as appropriate donor.

According to the data collected to date we propose a model for the cell cycle-dependent regulation of the DSB repair pathway choice (Figure 28). Cdk1 activity promotes DSB repair through HR by stimulating DSB end resection, which is necessary for HR and inhibitory for NHEJ (Aylon *et al.*, 2004; Ira *et al.*, 2004; Zhang *et al.*, 2009). In particular, Cdk1 triggers DSB resection by both counteracting the inhibitory effect of the NHEJ proteins and stimulating the activity of DSB resection machinery. In fact, it has been demonstrated that Cdk1 phosphorylates and therefore activates Sae2 (Huertas *et al.*, 2008), which is involved in the removal of Yku from DSB ends and in the initiation of DSB resection, and Dna2 (Chen *et al.*, 2011), that drives extensive DSB resection. This first level of Cdk1-dependent regulation on DSB repair is the only one required to carry out SSA and noncrossover recombination. However, in order to generate crossover recombination products, other levels of Cdk1-dependent regulation are required. For instance, Mms4 resolvase, that gives rise to crossover product formation by cleaving dHJ, has been recently demonstrated to be target of Cdk1 (Gallo-Fernández et al., 2012; Matos et al., 2011). Whether Cdk1 regulates other steps during HR in order to generate crossover products is still unknown.

Figure 28 – **DSB repair during the cell cycle.** The MRX complex and Yku bind to DSB ends. In G1 (**left panel**), Yku and MRX mediate recruitment of the NHEJ proteins (Lif1, Dnl4 and Nej1), which allow NHEJ-mediated religation of the DSB ends. Both Yku and the NHEJ proteins prevent initiation of resection by MRX. When the DSB ends are not bound by MRX, Yku still prevents Exo1- and Sgs1-mediated resection. In S/G2 (**right panel**), Cdk1 enhances Sae2/MRX function in resection by phosphorylating Sae2, thus channelling DNA repair into HR. Then, MRX and Sae2 catalyze the initial processing of the 5' strand, resulting in generation of short ssDNA stretches. Sae2 phoshorylation also promotes removal of Yku to allow further nucleolytic resection by the concerted action of Exo1 and Dna2 (together with Sgs1) at the DSB ends. Also Dna2 action requires phosphorylation by Cdk1. The 3'-ended ssDNA invades the homologous DNA sequence and the displaced strand anneals with the ssDNA on the other end of the break, forming a double Holliday Junction (dHJ). Resolution of the dHJ through nucleolytic cleavage by resolvases gives rise to crossover or noncrossover products. Among the resolvases, Mms4 is a known target of Cdk1 in promoting recombination.

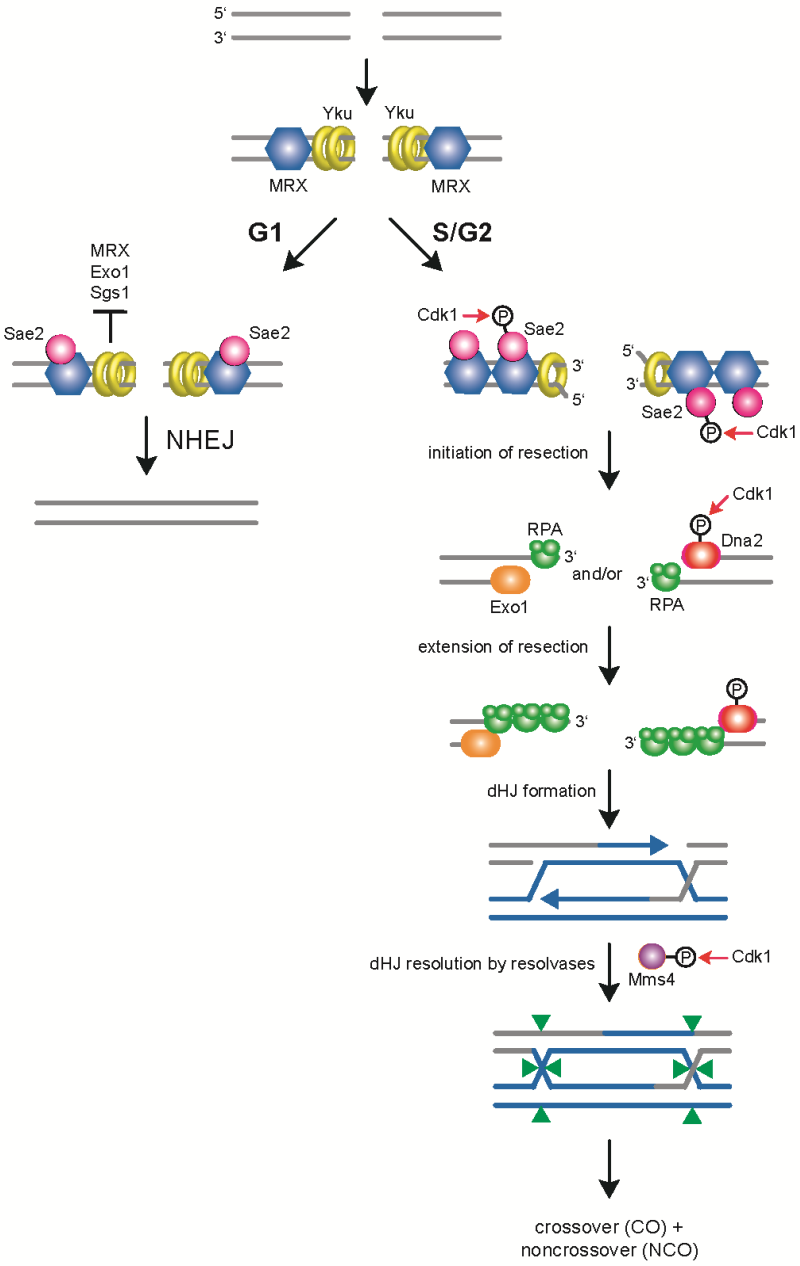

Figure 28 – **DSB repair during the cell cycle.**

Interplay between yeast Thioredoxin Reductase and Recombination Pathways in response to Replication Stress

Eukaryotic genome is particularly vulnerable during the S phase when the progression of replisome, the complex responsible for DNA synthesis, is hamper by the presence of a replication stress such as DNA lesions, DNA secondary structure, or abnormal levels or dNTPs (Branzei and Foiani, 2010). In all these situations it is important to stabilize the replisome on DNA in order to permit the resumption of DNA synthesis once the replication stress are removed (Tourrière and Pasero, 2007).

A failure to maintain the replisome bound to the DNA results in a so called fork collapse. This event could lead to the accumulation of unreplicated DNA regions and is frequently associated with DSB generation and genome instability. Several mechanisms cooperates to ensure replisome stability on DNA when replication fork stalls and to permit the resumption of DNA synthesis. Among these mechanism the S-phase checkpoint and homologous recombination play fundamental roles.

The S-phase checkpoint, or replication checkpoint, is required to stabilize replisome at stall replication forks and to block the cell cycle progression, by regulating the firing of late replication origins, the transcriptional induction of DNA damage response genes and inhibition of mitosis until replication is completed (Tercero et al., 2003, Figure 5).

HR is required to restore a functional replication fork after the collapse of the replisome from DNA, however several recent data suggest that this is not the only role of HR factors during DNA replication. In particular, Rad51 seems to assists DNA polymerase ε stably associated to DNA when replisome stalls (Bjergbaek et al., 2005). Moreover, it was proposed that MRX complex promotes the recruitment of cohesin to stalled replication forks which is necessary for resume DNA replication (Tittel -Elmer et al., 2012).

MRX deficient cells show phenotype that resemble to be link to replication defects like an high rate of chromosome rearrangements and sensitivity to the replication inhibitor hydroxyurea (HU). Like disruption of MRX complex also the lack of Rad51 causes sensitivity to HU in yeast (Tourrière and Pasero, 2007). In vertebrates both MRN complex and Rad51 protein are required for cells viability. Thus, several results suggest that both MRX/MRN and Rad51 play an important role during DNA replication.

In order to better understand the reasons why mutants affecting MRX functions are sensitive to replication stress we performed a genetic screening searching for extragenic suppressors of the HU sensitivity of *mre11Δ* strain. Finally, 27 mutants were confirmed as good suppressors of HU sensitivity. Among them we identified 12 recessive and 15 dominant or semi-dominant mutants. During this thesis we focused on the 12 recessive suppressors although it would be interesting also to study the dominants mutants whose suppression phenotype should be related to gain of function mutations.

We discover that all recessive mutants whose suppression was due to mutations in only one gene were mutated in *TRR1*. Thus, it is possible that the only gene whose loss of function alone is able to suppress the HU sensitivity of *mre11Δ* cells is *TRR1*. *TRR1* encode for cytoplamatic thioredoxin reductase, an enzyme very conserved during the evolution, which acts as a disulfide reductase in the major system required to maintain the proper intracellular redox state (Toledano et al., 2007). We discovered that our thioredoxin reductase mutants does not only suppress the HU sensitivity of MRX deficient cells but also that of other mutants like *rad51Δ, rad52Δ* and *sae2Δ.* As Rad51, Rad52 and Sae2 all participate in the homologous recombination we concluded that loss of function of Trr1 is able to suppress the HU sensitivity of recombination mutants.

Found a connection between thioredoxin reductase and HU sensitivity of recombination mutants was difficult as they are involved in processes apparently distant of cellular metabolism. However, despite thioredoxin system has been mainly associated with the response against oxidative stress it is becoming clear that this system functions also in other cellular pathways (Tonissen and Di Trapani, 2009). An example of the multifunctional role of thioredoxin system comes from mammals. High levels of thioredoxins and thioredoxin reductase are present in many different tumor types compared to levels observed in corresponding healthy cells from the same patient (Berggren et al., 1996; Lincoln et al., 2003). Moreover, results from various studies have suggested that Trx may have opposite functions in cancer cell depending on the stage of cancer development. At early stage Trx may be beneficial for preventing cancer due to its capability to counteract the oxidative stress caused by many carcinogens. Once a cell has initiated a cancer phenotype high levels of Trx may assist cancer development due to its growth promoting and antiapoptotic functions (Saitoh et al., 1998; Wakasugi et al., 1990; Welsh et ail., 2002). Another evidence which links thioredoxin system with genome stability comes from yeast. Indeed, it has been demonstrated that deletion of *TSA1* induces genome instability through activation of DNA damage checkpoint and elevated dNTPs (Iraqui et al., 2009; Tang et al., 2009). *TSA1* encodes for major yeast peroxiredoxin which is directly regulated at protein level from thioredoxins (Figure 7). Moreover, also the transcriptional regulator Yap1, which is activated in the absence of Trr1 positively regulates *TSA1* expression (Carmel-Harel et al., 2001).

Thus, the understanding of how the partial inactivation of Trr1 suppress the death in HU of recombination mutants could shed light into the interplays between homologous recombination and thioredoxin system functions during DNA replication stress.

Thioredoxin system were originally described as a hydrogen donor for ribonucleotide reductase (Laurent et al., 1964). Thus, thioredoxins are required for maintenance of proper dNTPs levels during S phase (Koc et al., 2006). Moreover it has been published that reduction of dNTPs amounts enhance DNA replication fidelity in vivo (Laureti et al., 2013) as high dNTPs levels are associate with genome instability (Chabes et al., 2003). Thus, we explored the possibility that a loss of function mutation in *TRR1* could influence DNA replication in *mre11Δ* cells by modulating dNTPs levels. However, neither the increase nor the decrease of dNTP levels affect the viability of *mre11Δ* cells on HU containing plates. These results suggest that the *trr1-2*-mediated suppression of recombination mutant HU sensitivity was not due to a modulation in dNTPs levels.

As most of known Trr1 functions pass through thioredoxins activities we test if also the HU sensitivity suppression of *mre11Δ* cells depends on *S. cerevisiae* Trx1 and Trx2. We demonstrated that neither singles nor the double deletions of *TRX1* and *TRX2* were able to suppress the HU sensitivity of *mre11Δ* cells. However, in agreement with a previous work which demonstrated that mutations in *TRR1* causes an increase in the expression of Trx2 (Carmel-Harel et al., 2001), we showed that also our *trr1-2* mutant presents high thioredoxins levels compared to wild type. However also the overexpression of both thioredoxins did not influence the HU sensitivity of recombination mutants. From these results we concluded that *trr1-2* suppresses the HU sensitivity independently from thioredoxin, raising the hypotesis of new role of thioredoxin reductase yet to be discover.

S phase checkpoint together with homologous recombination (HR) are required to maintain genome integrity during DNA replication by ensuring replisome stability and recovery after fork collapse (Li and Heyer, 2008; Tercero et al., 2003). We discovered that *trr1-2* was able to suppress the HU sensitivity of recombination mutants (*mre11Δ,*

rad51Δ, rad52Δ and *sae2Δ*) while it did not suppress the HU sensitivity of checkpoint mutants like *mec1Δ* and *mrc1Δ*. These results could suggest that checkpoint is required for *trr1-2* mediated suppression. This hypothesis is supported by the fact that MRX complex is required to properly activate the DNA damage checkpoint (D'Amours and Jackson, 2001). Moreover, it has been demonstrated that in *S. cerevisiae* exposure to low concentrations of H_2O_2 triggers DNA damage checkpoints specifically during S phase (Leroy et al., 2001). Thus, we postulated that loss of function in Trr1 causes an increase of intracellular amount of ROS which improves the checkpoint activation specifically during S-phase. This checkpoint reactivation could be responsible for the HU sensitivity suppression of recombination mutants. In order to test this hypothesis, we decided to check if mutation in *TRR1* improved checkpoint activation in *mre11Δ* and *rad51Δ* mutant during replication stress. We demonstrated that not only the deletion of *MRE11* but also that of *RAD51* lead to a defects in Rad53 phosphorylaton during HU treatment. Trr1-2 was able to improve checkpoint activation of both *mre11Δ* and *rad51Δ* cells raising the possibility that checkpoint reactivation was responsible for the replication stress suppression effect.

To test if this was the case we inserted the deletions of some checkpoint genes like *MEC1* and *MRC1* in the double mutants *mre11Δ trr1-2* and *rad51Δ trr1-2* in order to test the HU sensitivity of the resulting triple mutants. We discovered that checkpoint proteins were not required to Trr1-2 mediated suppression of recombination mutants HU sensitivity.

All together these results led us to conclude that mutations in *TRR1* are able to suppress the checkpoint activation defects typical of recombination mutants. However, the HU sensitivity suppression of recombination mutants is not due to checkpoint reactivation.

Has becoming clear that in response to replication stress, several HR factors are recruited at stalled forks. During HU treatment MRX complex is recruited at replication forks where stabilizes replisome components independently from its nuclease activity or S-phase checkpoint (Tittel-Elmer et al., 2009). In both yeast and vertebrate Rad51 assists continuous DNA synthesis by preventing degradation of nascent strands at stalled forks (Lambert et al., 2007; Petermann and Helleday, 2010; Petermann et al., 2010).

We showed that both *mre11Δ* and *rad51Δ* cells were able to recover DNA replication once released from HU treatment, but failed to divide nucleai. Mutations in *TRR1* increased the number of nuclear division events in *mre11Δ* and *rad51Δ* mutants released from HU. It has been demonstrated that MRX deficint cells treated with HU accumulate stretch of unreplicated DNA (Tittel-Elmer et al., 2009). Thus, also without a direct evidence, we speculate that mutations in *TRR1* decrease the amount of unreplicated DNA improving chromosome segragation and nuclear division in recombination mutants released from replication stress.

Since agents that stall or collapse replication forks, as hydroxyurea, thymidine and camptothecin strongly induce DSBs formation and homologous recombination (Arnaudeau et al., 2001; Lundin et al., 2002) it is possible that stretch of unreplicated DNA result from the inability of recombination mutants to process DNA lesions such as DSBs. Thus, we followed the DSB formation before and after HU treatment in *rad51Δ trr1-2* cells and in its controll strains demonstrating that mutations in *TRR1* decreased the percentage of cells with Rad52 foci both beore and after HU treatment. Altought we did not elucidate the specific mechanism trough which *trr1-2* positively influence the viability of replication mutants under HU treatment, we propose that, during replication stress, Trr1 promotes the formation of DNA lesions or structures which become substrate of recombination. Thus, partial inactivation of thioredoxin

reductase could prevents the formation of DNA structures which become substrates recombination or promotes HR alternative mechanisms. This leads to an improvement of nuclear division efficiency and cell viability of recombination mutants which undergo to replication stress.

MATERIALS AND METHODS

YEAST AND BACTERIAL STRAINS

Yeast strains. Yeast strains used for this work are listed in **Table 1**.

Table 1 - *Saccharomyces cerevisiae* strains used in this study.

Strains	Relevant genotype	Source
YMV86	*ho hmlΔ::ADE1 mataΔ::hisG hmrΔ::ADE1 ade3::GAL-HO ade1 lys5 ura3-52 trp1 leu2::leu2-NATMX-HOcs*	(Vaze *et al.*, 2002)
YLL2756	YMV86 *yku70Δ::URA3*	This study
YLL3047	YMV86 *CDC28-3HA::TRP1*	This study
YLL3048	YMV86 *yku70Δ::URA3 CDC28-3HA::TRP1*	This study
YMV45	*ho hmlΔ::ADE1 mataΔ::hisG hmrΔ::ADE1 leu2::leu2(Asp718-SalI)-URA3-HOcs ade3::GAL-HO ade1 lys5 ura3-52 trp1 bar1Δ::HPHMX*	(Vaze *et al.*, 2002)
YLL2912	YMV45 *bar1Δ::HPHMX yku70Δ::NATMX*	This study
YLL2910	YMV45 *bar1Δ::HPHMX rad9Δ::KANMX4*	This study
YLL2903	YMV45 *bar1Δ::HPHMX yku70Δ::NATMX rad9Δ::KANMX4*	This study
YLL3036	YMV45 *bar1Δ::HPHMX CDC28-3HA::TRP1*	This study
YLL3049	YMV45 *bar1Δ::HPHMX yku70Δ::NATMX CDC28-3HA::TRP1*	This study
YLL3050	YMV45 *bar1Δ::HPHMX rad9Δ::KANMX4 CDC28-3HA::TRP1*	This study
YLL3037	YMV45 *bar1Δ::HPHMX yku70Δ::NATMX rad9Δ::KANMX4 CDC28-3HA::TRP1*	This study
YLL2956	YMV45 *bar1Δ::HPHMX yku70Δ::NATMX rad9Δ::KANMX4 rad52Δ::TRP1*	This study
YLL3043	YMV45 *bar1Δ::HPHMX yku70Δ::NATMX rad9Δ::KANMX4 rad51Δ::TRP1*	This study
YLL3044	YMV45 *bar1Δ::HPHMX trp1::GAL-SIC1ntΔ::TRP1*	This study
YLL3045	YMV45 *bar1Δ::HPHMX yku70Δ::NATMX rad9Δ::KANMX4 trp1::GAL-SIC1ntΔ::TRP1*	This study
JKM139 *bar1Δ*	*MATa ho hmlΔ::ADE1 hmrΔ::ADE1 ade1-100 leu2-3;112 lys5 trp1::hisG ura3-52 ade3::GAL-HO bar1Δ::HPHMX*	(Lee *et al.*, 1998)

YLL2892	JKM139 *bar1Δ::HPHMX yku70Δ::URA3*	This study
YLL2962	JKM139 *bar1Δ::HPHMX rad9Δ::KANMX4*	This study
YLL2978	JKM139 *bar1Δ::HPHMX yku70Δ::URA3 rad9Δ::KANMX4*	This study
tGI354 *bar1Δ*	*ho hmlΔ::ADE1 MATa-inc hmrΔ::ADE1 ade1 leu2-3;112 lys5 trp1::hisG ura3-52 ade3::GAL::HO arg5,6::MATa::HPHMX bar1Δ::TRP1*	(Saponaro *et al.*, 2010)
YLL2954	tGI354 *bar1Δ::TRP1 yku70Δ::URA3*	This study
YLL2980	tGI354 *bar1Δ::TRP1 rad9Δ::KANMX4*	This study
YLL2970	tGI354 *bar1Δ::TRP1 yku70Δ::URA3 rad9Δ::KANMX4*	This study
YLL3019	tGI354 *bar1Δ::TRP1 yku70Δ::NATMX rad9Δ::KANMX4 ura3::GAL-SIC1ntΔ-MYC-HIS::URA3*	This study
YLL3051	tGI354 *bar1Δ::TRP1 CDC28-3HA::URA3*	This study
YLL3052	tGI354 *bar1Δ::TRP1 yku70Δ::NATMX rad9Δ::KANMX4 CDC28-3HA::URA3*	This study
YLL3038	tGI354 *bar1Δ::TRP1 ura3::GAL-CLB2dbΔ::URA3*	This study
YLL3039	tGI354 *bar1Δ::TRP1 yku70Δ::NATMX rad9Δ::KANMX4 ura3::GAL-CLB2dbΔ::URA3*	This study
YLL936.3	W303 *MATa mre11Δ::HIS3*	This study
SMS2	W303 *MATa mre11Δ::HIS3 trr1-2*	This study
DMP5426/1A	W303 *MATa mre11Δ::HIS3 trr1-2*	This study
SMS6	W303 *MATa mre11Δ::HIS3 trr1-6*	This study
SMS2A	W303 *MATa trr1-2*	This study
SMS6B	W303 *MATa trr1-6*	This study
YLL3149.1	W303 *MATa trr1Δ::KANMX4*	This study
DMP5427/1C	W303 *MATa mre11Δ::HIS3 trr1Δ::KANMX4*	This study
YLL1067.4	W303 *MATa rad51Δ::HIS3*	This study
DMP5646/8B	W303 *MATa rad51Δ::HIS3 trr1-2*	This study
DMP5646/10C	W303 *MATa rad51Δ::HIS3 trr1-2*	This study
YLL1068.1	W303 *MATa rad52Δ::TRP1*	This study
DMP5576/3D	W303 *MATa rad52Δ::TRP1 trr1-2*	This study
DMP5576/6B	W303 *MATa rad52Δ::TRP1 trr1-2*	This study
YLL1069.3	W303 *MATa sae2Δ::KANMX4*	This study
DMP5580/2B	W303 *MATa sae2Δ::KANMX4 trr1-2*	This study
DMP5580/1C	W303 *MATa sae2Δ::KANMX4 trr1-2*	This study
YLL1310.1	W303 *MATa mrc1Δ::HIS3*	This study

DMP5579/1A	W303 *MATa mrc1Δ::HIS3 trr1-2*	This study
DMP5579/2B	W303 *MATa mrc1Δ::HIS3 trr1-2*	This study
YLL2415.1	W303 *MATa sgs1Δ::URA3*	This study
DMP5578/1A	W303 *MATa sgs1Δ::URA3 trr1-2*	This study
DMP5401/4A	W303 *MATa sml1Δ::KANMX4 trr1-2*	This study
DMP5402/3B	W303 *MATa sml1Δ::KANMX4 trr1-6*	This study
DMP2854/2B	W303 *MATa sml1Δ::KANMX4 mec1Δ::HIS3*	This study
DMP5401/12A	W303 *MATa sml1Δ::KANMX4 mec1Δ::HIS3 trr1-2*	This study
DMP5402/8D	W303 *MATa sml1Δ::KANMX4 mec1Δ::HIS3 trr1-6*	This study
DMP2855/7C	W303 *MATa sml1Δ::KANMX4 rad53Δ::HIS3*	This study
DMP5403/1C	W303 *MATa sml1Δ::KANMX4 rad53Δ::HIS3 trr1-2*	This study
DMP5404/4C	W303 *MATa sml1Δ::KANMX4 rad53Δ::HIS3 trr1-6*	This study
DMP5704/13B	W303 *MATa sml1Δ::KANMX4 rad51Δ::HIS3*	This study
DMP5704/13A	W303 *MATa sml1Δ::KANMX4 rad51Δ::HIS3 trr1-2*	This study
DMP5704/17B	W303 *MATa sml1Δ::KANMX4 mec1Δ::HIS3 rad51Δ::HIS3*	This study
DMP5704/15A	W303 *MATa sml1Δ::KANMX4 mec1Δ::HIS3 rad51Δ::HIS3 trr1-2*	This study
YLL2905.2	W303 W303 *MATa ixr1Δ::KANMX4*	This study
DMP5451/1D	W303 W303 *MATa ixr1Δ::KANMX4 mre11Δ::HIS3*	This study
DMP5451/2A	W303 W303 *MATa ixr1Δ::KANMX4 mre11Δ::HIS3*	This study
DMP2818/1B	W303 *MATa sml1Δ::KANMX4*	This study
DMP5426/1C	W303 *MATa sml1Δ::KANMX4 mre11Δ::HIS3*	This study
DMP5426/4A	W303 *MATa sml1Δ::KANMX4 mre11Δ::HIS3*	This study
YLL3155.9	W303 *MATa trx1Δ::NAT*	This study
YLL3156.3	W303 *MATa trx2Δ::HPH*	This study
DMP5425/1A	W303 *MATa trx1Δ::NAT trx2Δ::HPH*	This study
DMP5455/2B	W303 *MATa trx1Δ::NAT mre11Δ::HIS3*	This study
DMP5455/1B	W303 *MATa trx2Δ::HPH mre11Δ::HIS3*	This study
DMP5455/3D	W303 *MATa trx1Δ::NAT trx2Δ::HPH mre11Δ::HIS3*	This study
DMP5789/2D	W303 *MATa rad51Δ::HIS3 trx1Δ::NAT*	This study
DMP5789/5B	W303 *MATa rad51Δ::HIS3 trx1Δ::NAT*	This study
DMP5789/3D	W303 W303 *MATa rad51Δ::HIS3 trx2Δ::HPH*	This study
DMP5789/10C	W303 W303 *MATa rad51Δ::HIS3 trx2Δ::HPH*	This study
DMP5790/2A	W303 *MATa rad51Δ::HIS3 trx1Δ::NAT trx2Δ::HPH*	This study
DMP5790/4C	W303 *MATa rad51Δ::HIS3 trx1Δ::NAT trx2Δ::HPH*	This study
YLL3363.1	W303 *MATa TRX2-HA::URA3*	This study

YLL3364.13	W303 *MATa TRX1-HA::URA3*	This study
DMP5627/1B	W303 *MATa RAD52-YFP*	This study
DMP5655/1D	W303 *MATa rad51Δ::HIS3 RAD52-YFP*	This study
DMP5655/1D	W303 *MATa trr1-2 RAD52-YFP*	This study
DMP5655/14C	W303 *MATa rad51Δ::HIS3 trr1-2 RAD52-YFP*	This study
DMP5780/4A	W303 *MATa rad51Δ::HIS3 sgs1Δ::URA3 trr1-2*	This study
DMP5780/7B	W303 *MATa rad51Δ::HIS3 sgs1Δ::URA3 trr1-2*	This study
DMP5780/1C	W303 *MATa rad51Δ::HIS3 sgs1Δ::URA3*	This study
DMP5652/18C	W303 *MATa rad51Δ::HIS3 mrc1Δ::URA3*	This study
DMP5652/11A	W303 *MATa rad51Δ::HIS3 mrc1Δ::URA3 trr1-2*	This study
DMP5652/12C	W303 *MATa rad51Δ::HIS3 mrc1Δ::URA3 trr1-2*	This study

Strains JKM139, YMV86 and YMV45 were kindly provided by J. Haber (Brandeis University, Waltham, USA). Strains YMV86 and YMV45 are isogenic to YFP17 (*matΔ::hisG hmlΔ::ADE1 hmrΔ::ADE1 ade1 lys5 ura3-52 trp1 ho ade3::GAL-HO leu2::cs*) except for the presence of a *LEU2* fragment inserted, respectively, 0.7 kb or 4.6 kb centromere-distal to *leu2::cs* (Vaze *et al.*, 2002). Strain tGI354 was kindly provided by G. Liberi (IFOM, Milano, Italy) and J. Haber (Saponaro *et al.*, 2010). Other strains were derivatives of W303 (*ade2-1*, *trp1-1*, *leu2-3,112*, *his3-11,15*, *ura3*, and *rad5-535*). To induce a persistent G1 arrest with α-factor, all strains used in this study carried the deletion of the *BAR1* gene, which encodes a protease that degrades the α-factor. Gene deletions were generated by one-step gene replacement. YMV86, YMV45, tGI354 and W303 derivatives strains carrying a fully functional *CDC28-HA, TRX1-HA, TRX2-HA, TRR1-MYC, trr1-2-MYC* and *trr1-6-MYC* allele at the corresponding chromosomal locus were generated by one-step PCR tagging method. A plasmid carrying the *GAL-CLB2dbΔ* allele was kindly provided by R. Visintin (IEO, Milan, Italy) and was used to integrate the *GAL-CLB2dbΔ* fusion at the *URA3* locus in the tGI354 derivative strains. Strain YLL3019, carrying the *GALSIC1ntΔ* allele integrated at the *URA3* locus, was obtained by transforming strain tGI354 *rad9Δ yku70Δ* with ApaI-

digested plasmid pLD1, kindly provided by J. Diffley (Clare Hall Laboratories, South Mimms, United Kingdom). The *GAL-SIC1ntΔ* fusion was cloned into a *TRP1*-based integrative plasmid that was used to integrate the fusion at the *TRP1* locus in the YMV45 derivative strains. Integration accuracy was verified by Southern blot analysis. Cells were grown in YEP medium (1% yeast extract, 2% bactopeptone) supplemented with 2% raffinose (YEP+raf) or 2% raffinose and 3% galactose (YEP+raf+gal).

E. coli strain *E. coli* DH5α™ strain *(F-,* ϕ80 *dlacZM15, D(lacZTA-argF) U169, deoR, recA1, endA1, hsdR17, (rK-,mK+) phoA supE44,* λ–, *thi-1, gyrA96, relA1)* is used as bacterial host for plasmid manipulation and amplification. *E. coli* DH5α™ competent cells are purchased from Invitrogen.

GROWTH MEDIA

S. cerevisiae media : YEP (Yeast-Extract Peptone) is the standard rich media for *S. cerevisiae* and contains 10 g/L yeast extract, 20 g/L peptone and 50 mg/L adenine. YEP must be supplemented with 2% glucose (YEPD), 2% raffinose (YEP+raf) or 2% raffinose and 2% galactose (YEP+raf+gal) as carbon source. YEP-based selective media are obtained including 400 μg/mL G418, 300 μg/mL hygromicin-B or 100 μg/mL nourseotricin. Solid media are obtained including 2% agar. Stock solutions are 50% glucose, 30% raffinose, 30% galactose, 80 mg/mL G418, 50 mg/mL hygromicin-B and 50 mg/mL nourseotricin. YEP and glucose stock solution are autoclave-sterilized and stored at RT. Sugars and antibiotics stock solutions are sterilized by micro-filtration and stored at RT and 4°C respectively. **S.C. (Synthetic Complete)** is the minimal growth media for *S. cerevisiae* and contains 1.7 g/L YNB (without aminoacids), 5 g/L

ammonium sulphate, 200µM inositol, 25 mg/L uracil, 25 mg/L adenine, 25 mg/L hystidine, 25 mg/L leucine, 25 mg/L tryptophan. S.C. can be supplemented with drop-out solution (20 mg/L arginine, 60 mg/L isoleucine, 40 mg/L lysine, 10 mg/L methionine, 60 mg/L phenylalanine, 50 mg/L tyrosine) based on ueast strains requirements. Different carbon sources can be used as in rich media (2% glucose, 2% raffinose or 2% raffinose and 1% galactose). Different carbon sources can be used as in rich media. One or more aminoacid/base can be omitted to have S.C.-based selective media (e.g. S.C.-ura is S.C. lacking uracil). To obtain G418 or NAT S.C. selective medium the 5 g/L ammonium sulphate are replaced with 1 g/L monosodic glutamic acid. Solid media are obtained by including 2% agar. Stock solutions are 17 g/L YNB + 50 g/L ammonium sulphate (or 10g/L monosodic glutamic acid), 5 g/L uracil, 5 g/L adenine, 5 g/L hystidine, 5 g/L leucine, 5 g/L tryptophan, 100X drop out solution (2 g/L arginine, 6 g/L isoleucine, 4 g/L lysine, 1 g/L methionine, 6 g/L phenylalanine, 5 g/L tyrosine), 20mM inositol. All of these solutions are sterilized by micro-filtration and stored at 4°C.

VB sporulation medium contains 13.6 g/L sodium acetate, 1.9 g/L KCl, 0.35 g/L MgSO4, 1.2 g/L NaCl. pH is adjusted to 7.0. To obtain solid medium include 2% agar. pH is adjusted to 7.0. Sterilization by autoclavation.

***E. coli* MEDIA : LD** is the standard growth medium for *E. coli*. LD medium contains 10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl. Solid medium is obtained by including 1% agar. LD+Amp selective medium is obtained including 50 µg/mL ampicillin. LD is autoclave-sterilized and stored at RT. Ampicillin stock solution (2.5 g/L) is sterilized by micro-filtration and stored at 4°C.

Conservation and storage of *S. cerevisiae* and *E. coli* strains: Yeast cells are grown 2-3 days at 30°C on YEPD plates, resuspended in 15% glycerol and stored at -80°C. Bacteria are grown o/n at 37°C on LD+Amp plates, resuspended in 50% glycerol and stored at -80°C. Yeast and bacteria cells can be stored for years in these conditions.

MOLECULAR BIOLOGY TECHNIQUES

Agarose gel electrophoresis: Agarose gel elctrophoresis is the most easy and common way of separating and analyzing DNA molecules. This technique allows the separation of DNA fragments based on their different molecular weight (or length in kb). The purpose of this technique might be to visualize the DNA, to quantify it or to isolate a particular DNA fragment. The DNA is visualized by the addition in the gel of ethidium bromide, which is a fluorescent dye that intercalates between bases of nucleic aicds. Ethidium bromide absorbs UV light and transmits the energy as visible orange light, revealing the DNA molecules to which is bound.

To pour a gel, agarose powder is mixed with TAE (0.04M Tris-Acetate 0.001M EDTA) to the desired concentration, and the solution is microwaved until completely melted. Most gels are made between 0.8% and 2% agarose. A 0.8% gel will show good resolution of large DNA fragments (5-10 Kb) and a 2% gel will show good resolution for small fragments (0.2-1 Kb). Ethidium bromide is added to the gel at a final concentration of 1 µg/mL to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 60°C, it is poured into a casting tray containing a sample comb and allowed to solidify at RT or at 4°C. The comb is then removed and the gel is placed into an electrophoresis chamber and just covered with the buffer (TAE). Sample containing DNA mixed with loading buffer are then pipetted into the sample

wells. The loading buffer contains 0.05% bromophenol blue and 5% glycerol, which give color and density to the sample. A marker containing DNA fragments of known length and concentration is loaded in parallel to determine size and quantity of DNA fragments in the samples. Then current is applied and DNA will migrate toward the positive electrode. When adequate migration has occurred, DNA fragments are visualized by placing the gel on a UV transilluminator.

DNA extraction from agarose gels (paper strip method): This method allow to isolate a DNA fragment of interest. Using a scalpel blade cut a slit immediately in front of the band to be extracted. Cut a piece of GF-C filter to size to fit inside the slit. Place the paper strip in the slit and switch on the current for 1-2 minutes at 150 V. The DNA runs onward into the paper and is delayed in the smaller mesh size of the paper. Remove the strip of paper and place it into a 0.5 mL microcentrifuge tube. Make a tiny hole in the bottom of the tube using a syringe needle, place the 0.5 mL tube inside a 1.5 mL tube and spin for 30 seconds. Buffer and DNA are retained in the 1.5 mL tube. Extract the DNA with 1 volume of phenol/chloroform and precipitate the DNA with 100mM sodium acetate and 3 volumes of 100% ethanol. After microcentrifugation re-dissolve DNA in an appropriate volume of water, TRIS (10mM Tris HCl pH 8.5) or TE (10mM Tris HCl 1mM EDTA pH7.4) buffer.

Restriction endonucleases: Type II endonucleases (also known as restriction endonuceases or restriction enzymes) cut DNA molecules at defined positions close to their recognitions sequences in a reaction known as enzymatic digestion. They produce discrete DNA fragments that can separated by agarose gel electrophoresis, generating distinct gel banding patterns. For these reasons they are used for DNA analysis and gene cloning. Restriction enzymes are generally stored at -20°C in a

solution containing 50% glycerol, in which they are stable but not active. Glycerol concentration in the reaction mixture must be below 5% in order to allow enzymatic reaction to occur. They generally work at 37°C with some exceptions (e.g. ApaI activity is maximal at 25°C) and they must be supplemented with a reaction buffer provided by the manufacturer, and in some cases with Bovin Serum Albumin. We use restriction endonucleases purchased from NEB and PROMEGA.

Ligation: DNA is previously purified from agarose gel with the paper strip method, phenol/chloroform extracted, ethanol precipitated and resuspended in the appropriate volume of water or TE buffer. The ligation reaction is performed in the following conditions: DNA fragment and vector are incubated overnight at 16°C with 1 µl T4 DNA Ligase (PROMEGA) and T4 DNA Ligase Buffer (PROMEGA). The ligation reaction is then used to transform competent *E. coli* cells. Plasmids are recovered from Amp+ transformants and subjected to restriction analysis.

Preparation of yeast genomic dna for Polymerase Chain Reaction: Resuspend yeast cells in 200 µL Yeast Lysis Buffer (2% TRITON X100, 1% SDS, 100mM NaCl, 10mM Tris HCl pH 8, 1mM EDTA pH 8), add 200 µL glass beads, 200 µL phenol/chloroform and vortex 3 minutes. Ethanol precipitate the aqueous phase obtained after 5 minutes centrifugation. Resuspend DNA in the appropriate volume of water and use 1 µL as a template for PCR.

Polymerase Chain Reaction (PCR): PCR allows to obtain high copy number of a specific DNA fragment of interest starting from very low quantity of DNA fragment. The reaction is directed to a specific DNA fragment by using a couple of oligonucleotides flanking the DNA sequence of interest. These oligonucleotides work

as primers for the DNA polymerase. The reaction consist of a number of polymerization cycles which are based on 3 main temperature-dependent steps: denaturation of DNA (which occur over 90°C), primer annealing to DNA (typically take place at 45-55°C depending on primer characteristic), synthesis of the DNA sequence of interest by a thermophilic DNA polymerase (which usually works at 68 or 72°C). Different polymerases with different properties (processivity, fidelity, working temperature, etc) are commercially available and suitable for different purpose. Taq polymerase works at 72°C and is generally used for analytical PCR. Polymerases with higher fidelity like Pfx and VENT polymerases, which work respectively at 68 and 72°C, are generally employed when 100% polymerization accuracy is required.

The typical 50 µL PCR mixture contains 1µL of template DNA, 0.5 µM each primer, 200µM dNTPs, 5 µL of 10X Reaction Buffer, 1mM $MgCl_2$, 1-2 U DNA polymerase and water to 50 µL. The typical cycle-program for a reaction is: 1. 3' denaturation at 94-95°C; 2. 30" denaturation at 94-95°C; 3. 30" annealing at primers Tm (melting temperature); 4. 1' polymerization per Kb at 68 or 72°C (depending on polymerase); 5. repeat 30 times from step 2; 6. 5-10' polymerization at 68-72°C. The choice of primer sequences determines the working Tm, which depends on the length (L) and GC% content of the oligonucleotides and can be calculated as follows: Tm = 59.9 + 0.41(GC%) – 675/L.

Plasmid dna extraction from *E. coli* (i): minipreps boiling: *E. coli* cells (2mL overnight culture) are harvested by centrifugation and resuspended in 500 µL STET buffer (8% sucrose, 5% TRITON X-100, 50mM EDTA, 50mM Tris-HCl, pH 8). Bacterial cell wall is digested boiling the sample for 2 minutes with 1 mg/mL lysozyme. Cellular impurities are removed by centrifugation and DNA is precipitated with isopropanol and resuspended in the appropriate volume of water or TE.

Plasmid DNA extraction from *E. coli* (ii): minipreps with Qiagen columns: This protocol allows the purification of up to 20 µg high copy plasmid DNA from 1-5 mL overnight *E. coli* culture in LD medium. Cells are pelleted by centrifugation and resuspended in 250 µL buffer P1 (100 µg/mL RNase, 50mM Tris HCl pH 8, 10mM EDTA pH 8). After addition of 250 µL buffer P2 (200mM NaOH, 1% SDS) the solution is mixed thoroughly by inverting the tube 4-6 times, and the lysis reaction occur in 5 minutes at RT. 350 µL N3 buffer (QIAGEN) are added to the solution, which is then centrifuged for 10 minutes. The supernatant is applied to a QIAprep spin column which is washed once with PB buffer (QIAGEN) and once with PE buffer (QIAGEN). The DNA is eluted with EB buffer (10mM Tris HCl pH 8.5) or water.

Transformation of *E.coli* DH5α: DH5α competent cells are thawed on ice. Then, 50-100 µL cells are incubated 30 minutes in ice with 1 µL plasmid DNA. Cells are then subjected to heat shock at 37°C for 30 seconds and then incubated on ice for 2 minutes. Finally, 900 µL LD are added to the tube and cells are incubated 30 minutes at 37°C to allow expression of ampicillin resistance. Cells are then plated on LD+amp and overnight incubated at 37°C.

Transformation of *S. cerevisiae*: YEPD exponentially growing yeast cells are harvested by centrifugation and washed with 1 mL 1M lithium acetate (LiAc) pH 7.5. Cells are then resuspended in 1M LiAc pH 7.5 to obtain a cells/LiAc 1:1 solution. 12 µL cells/LiAc are incubated 30-45 minutes at RT with 45 µL 50% PEG (PolyEthyleneGlycol) 3350, 4 µL carrier DNA (salmon sperm DNA) and 1-4 µL DNA of interest (double each quantity when transform with PCR products). After addition of 6 µL 60% glycerol cells are incubated at RT for 30-45 minutes, heat-shocked at 42°C for 5-10 minutes and plated on appropriate selective medium.

Extraction of yeast genomic dna (teeny yeast dna preps): Yeast cells are harvested from overnight cultures by centrifugation, washed with 1 mL of 0.9M sorbytol 0.1M EDTA pH 7.5 and resuspended in 0.4 mL of the same solution supplemented with 14mM β-mercaptoethanol. Yeast cell wall is digested by 45 minutes incubation at 37°C with 0.4 mg/mL 20T zimoliase. Spheroplasts are harvested by 30 seconds centrifugation and resuspended in 400 µL TE. After addition of 90 µL of a solution containing EDTA pH 8.5, Tris base and SDS, spheroplasts are incubated 30 minutes at 65°C. Samples are kept on ice for 1 hour following addition of 80 µL 5M potassium acetate. Cell residues are eliminated by 15 minutes centrifugation at 4°C. DNA is precipitated with chilled 100% ethanol, resuspended in 500 µL TE and incubated 30 minutes with 25 µL 1 mg/mL RNase to eliminate RNA. DNA is then precipitated with isopropanol and resuspended in the appropriate volume (typically 50 µL) of TE.

Southern blot analysis: Yeast genomic DNA prepared with standard methods is digested with the appropriate restriction enzyme(s). The resulting DNA fragments are separated by agarose gel electrophoresis in a 0.8% agarose gel. When adequate migration has occurred, gel is washed 40 minutes with a denaturation buffer (0.2N NaOH, 0.6M NaCl), and 40 minutes with a neutralization buffer (1.5M NaCl, 1M Tris HCl, pH 7.4). DNA is blotted onto a positively charged nylon membrane by overnight capillary transfer with 10X SSC buffer (20X SSC: 3M sodium chloride, 0.3M sodium citrate, pH 7.5). Membrane is then washed with 4X SSC and UV-crosslinked. Hybridization is carried out by incubating membrane for 5 hours at 50°C with pre-hybridization buffer (50% formamide, 5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 2% Blocking reagent) following by o/n incubation at 50°C with pre-hybridization buffer + probe. The probe is obtained by random priming method (DECAprime™ kit by Ambion) on a suitable DNA template and with ^{32}P d-ATP. Filter is then washed

(45'+15') at 55°C with a washing solution (0.2M sodium phosphate buffer pH 7.2, SDS 1%, water), air dried and then exposed to an autoradiography film.

Denaturing gel electrophoresis and southern blot analysis to visualize ssDNA: A 0.8% agarose gel (in H_2O) is submerged in a gel box containing a 50mM NaOH, 1mM EDTA solution for 30 minutes to equilibrate. Ethidium bromide is omitted because it does not efficiently bind to DNA under these conditions. After digestion with the appropriate restriction enzyme(s), DNA samples are prepared by adjusting the solution to 0.3M sodium acetate and 5mM EDTA (pH 8.0) following by addition of 2 volumes of ethanol to precipitate DNA. After chilling (o/n) and centrifuging the samples (15 minutes, possibly at 4°C), pellet is resuspended in alkaline gel loading buffer (1X buffer: 50mM NaOH, 1mM EDTA pH 8.5, 2.5% Ficoll (Type 400) and 0.025% bromophenol blue). After loading the DNA in the gel, a glass plate can be placed on the gel to prevent the dye from diffusing from the agarose during the course of the run. Because of the large currents that can be generated with denaturing gels, gels are usually run slowly at lower voltages (e.g. 30 V o/n). After the DNA has migrated far enough, the gel can be stained with 0.5 µg/ml ethidium bromide in 1X TAE electrophoresis buffer (1 hour). The DNA will be faint because the DNA is single stranded. Gel is then soaked in 0.25N HCl for 7 minutes with gentle agitation, rinsed with water and soaked in 0.5M NaOH, 1.5M NaCl for 30 minutes with gentle agitation. Gel is then rinsed briefly with water and DNA is blotted by capillary transfer onto neutral nylon membrane using 10X SSC. Hybridization is carried out by incubating membrane for 5 hours at 42°C with pre-hybridization buffer (50% formamide, denhardts solution + 4X BSA, 6%

destran sulphate, 100 µg/mL salmon sperm DNA, 200 µg/mL tRNA carrier) following by o/n incubation at 42°C with pre-hybridization buffer + ssRNA probe. The ssRNA probe is obtained by *in vitro* transcription using Promega Riboprobe System-T7 and a pGEM-7Zf-based plasmid as a template. Following hybridization, membrane is washed twice with 5X SSPE (20X SSPE = 3M NaCl, 200µM NaH2PO4, 20µM EDTA, pH 7.4) at 42°C for 15 minutes, 30 minutes with 1X SSPE 0.1% SDS at 42°C, 30 minutes with 0.1X SSPE 0.1% SDS at 42°C, 15 minutes with 0.2X SSPE 0.1% SDS at 68°C and 5 minutes with 0.2X SSPE at RT. Finally membrane is exposed to a X-ray film.

SYNCHRONIZATION OF YEAST CELLS

Synchronization of yeast cells with α-factor: α-factor allows to synchronize a population of yeast cells in G1 phase. This pheromone activates a signal transduction cascade which arrests yeast cells in G1 phase. Only *MATa* cells are responsive to α-factor. To synchronize in G1 a population of exponentially growing yeast cells in YEPD, 2 µg/mL α-factor is added to 6 x 10^6 cells/mL culture. As the percentage of budded cells will fall below 5% cells are considered to be G1-arrested. Cells are then washed and resuspended in fresh medium with or without 3 µg/mL α-factor to keep cells G1-arrested or release them into the cell cycle respectively. At this time cell cultures can be either treated with genotoxic agents or left untreated. If cells carry the deletion of *BAR1* gene, that encodes a protease that degrades the α-factor, 0.5 µg/mL α-factor is sufficient to induce a G1-arrest that lasts several hours.

Synchronization of yeast cells with nocodazole: Nocodazole allows to synchronize a population of yeast cells in G2 phase. This drug causes the depolimerization of microtubules, thus activating the mitotic checkpoint which arrests cells at the metaphase to anaphase transition (G2 phase). To synchronize in G2 a population of exponentially growing yeast cellsin YEPD, 0.5 µg/mL nocodazole is added to 6 x 10^6 cells/mL culture together with DMSO at a final concentration of 1% (use a stock solution of 100X nocodazole in 100% DMSO). As the percentage of dumbbell cells will reach 95% cells are considered to be G2-arrested. Cells are then washed and resuspended in fresh medium with or without 1.5 µg/mL nocodazole to keep cells G2-arrested or release them into the cell cycle respectively. At this time cell cultures can be either treated with genotoxic agents or left untreated.

OTHER TECHNIQUES

FACS analysis of DNA contents: FACS (Fluorescence-Activated Cell Sorting) analysis allow to determine the DNA content of every single cell of a given population of yeast cells. 6 x 10^6 cells are harvested by centrifugation, resuspended in 70% ethanol and incubated at RT for 1 hour. Cells are then washed with 1 mL 50mM Tris pH 7.5 and incubated overnight at 37°C in the same solution with 1 mg/mL RNase. Samples are centrifuged and cells are incubated at 37°C for 30 minutes with 5 mg/mL pepsin in 55mM HCl, washed with 1 mL FACS Buffer and stained in 0.5 mL FACS buffer with 50 µg/mL propidium iodide. 100 µL of each sample are diluted in 1 mL 50mM Tris pH 7.5 and analyzed with a Becton-Dickinson FACS-Scan. The same samples can also be analyzed by fluorescence microscopy to score nuclear division.

Total protein extracts: Total protein extracts were prepared from 10^8 cells collected from exponentially growing yeast cultures. Cells are harvested by centrifugation and

washed with 20% trichloracetic acid (TCA) in order to prevent proteolysis and resuspended in 50 µL 20% TCA. After addition of 200 µL of glass beads, cells are disrupted by vortexing for 8 minutes. Glass beads are washed with 400 µL 5% TCA, and the resulting extract are centrifuged at 3000 rpm for 10 minutes. The pellet is resuspended in 70 µL Laemmli buffer (0.62M Tris, 2% SDS, 10% glycine, 0.001% Bfb, 100mM DTT), neutralized with 30 µL 1M Tris base, boiled for 3 minutes, and finally clarified by centrifugation.

SDS-PAGE and western blot analysis: Protein extracts are loaded in 10% polyacrylamide gels (composition). Proteins are separated based on their molecular weight by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE). When adequate migration has occurred proteins are blotted onto nitrocellulose membrane. Membrane is saturated by 1 hour incubation with 4% milk in TBS containing 0.2% TRITON X-100 and incubated for 2 hours with primary antibodies. Membrane is washed three times with TBS for 10 minutes, incubated for 1 hour with secondary antibodies and again washed with TBS. Detection is performed with ECL (Enhanced ChemiLuminescence – GE Healthcare) and X-ray films according to the manufacturer. Primary polyclonal
rabbit anti-Rad53 antibodies are kindly provided by John Diffley (Clare Hall Laboratories, London). Primary monoclonal 12CA5 anti-HA and 9E10 anti-MYC antibodies are purchased at GE Healthcare, as well as peroxidase conjucated IgG anti-rabbit and anti-mouse secondary antibodies.

Kinase assay: For Cdk1 kinase assays, protein extracts were prepared as described previously (Schwob *et al.*, 1994). HA-tagged Cdk1 was immunoprecipitated with anti-HA antibody from 150 µg of protein extracts and the kinase activity in the immunoprecipitates was measured on histone H1 (Surana et al., 1993).

DSB resection and repair: DSB formation and repair in YMV86 and YMV45 strains were detected by Southern blot analysis using an Asp718-SalI fragment containing part of the *LEU2* gene as a probe. DSB end resection at the *MAT* locus in JKM139 derivative strains was analyzed on alkaline agarose gels as described in (Clerici *et al.*, 2008), by using a single-stranded probe complementary to the unresected DSB strand. This probe was obtained by *in vitro* transcription using Promega Riboprobe System-T7 and plasmid pML514 as a template. Plasmid pML514 was constructed by inserting in the pGEM7Zf EcoRI site a 900-bp fragment containing part of the *MATα* locus (coordinates 200870 to 201587 on chromosome III). Quantitative analysis of DSB resection was performed by calculating the ratio of band intensities for ssDNA and total amount of DSB products. DSB repair in tGI354 strain was detected as described in (Saponaro *et al.*, 2010). To determine the amount of noncrossover and crossover products, the normalized intensity of the corresponding bands at different time points after DSB formation was divided by the normalized intensity of the uncut *MATa* band at time zero before HO induction (100%). The repair efficiency (NCO+CO) was normalized with respect to the efficiency of DSB formation by subtracting the value calculated 2 hours after HO induction (maximum efficiency of DSB formation) from the values calculated at the subsequent time points after galactose addition.

Screening for suppressors of the hu sensitivity of *mre11Δ* cells: We searched for spontaneous extragenic mutations suppressing the HU sensitivity of *mre11Δ* cells. Since 40mM HU was the minimal HU dose impairing the ability of *mre11Δ* cells to form colonies, we plated *mre11Δ* (YLL936.3) cells on yeast extract-peptone-dextrose (YEPD) plates containing 40mM HUand searched for clones able to form colonies. This analysis allowed us to identify 27 independent clones able to grow on 40mM HU. By

crossing these clones with a *mre11Δ* strain, we found that the suppressor phenotype for twelve of them was due to a recessive mutations. By crossing these clones with a *MRE11* strain, we found that the suppressor phenotype for two of them was due to a single-gene recessive mutation. Both these two clones was also temperature sensitive for growth, and this phenotype segregated tightly linked to the suppressor phenotype. By complementation analysis we determined that the two clones were mutated in the same gene. We cloned the corresponding gene by transforming the original mutant clone with a yeast genomic DNA library constructed in a *LEU2* centromeric plasmid and searching for recombinant plasmids able to allow the mutant to form colonies at the restrictive temperature of 37°C. Analysis of several positive transformant clones revealed that the minimal complementing region was restricted to a DNA fragment containing the *TRR1* gene. Further genetic analysis allowed us to demonstrate that *TRR1* was indeed the gene identified by the suppressor mutation.

Microscopy: To visualize Rad52-YFP foci, cells expressing *RAD52-YFP* were grown in synthetic medium supplemented with adenine to minimize autofluorescence. The cells were washed in 0.1 M potassium phosphate buffer at the time points of interest and analyzed immediately at the microscope. Cells were imaged on concanavalin A-coated slides. Microscopy was performed on a Leica TCS resonant STED DMI6000 CS microscope equipped with a multiline argon ion laser. Images of the YFP-stained yeast cells were acquired by collecting between 530 and 600 nm the fluorescence excited by the 27-µW output of the 514-nm line of the argon laser. Both the emission and the transmitted light images have been recorded at 400-Hz scan speed through a 100X HCX PL APO oil objective (numerical aperture = 1.4) after identification of the cellular

focal plane by 1-µm step z-scan measurements. Microscopy images were analyzed by using ImageJ.

Drop test: For spot assays, exponentially growing overnight cultures were counted, and 10-fold serial dilutions of equivalent cell numbers were spotted onto plates containing the indicated media. Experiments involving G1 synchronization were carried out by incubating exponentially growing cells in appropriate media containing 5 µg of α-factor/ml at 25°C for 2 h.

REFERENCES

Aboussekhra, A., Chanet, R., Zgaga, Z., Cassier-Chauvat, C., Heude, M., and Fabre, F. (1989). RADH, a gene of Saccharomyces cerevisiae encoding a putative DNA helicase involved in DNA repair. Characteristics of radH mutants and sequence of the gene. Nucleic Acids Res. *17*, 7211–7219.

Admire, A., Shanks, L., Danzl, N., Wang, M., Weier, U., Stevens, W., Hunt, E., and Weinert, T. (2006). Cycles of chromosome instability are associated with a fragile site and are increased by defects in DNA replication and checkpoint controls in yeast. Genes Dev. *20*, 159–173.

Alcasabas, A.A., Osborn, A.J., Bachant, J., Hu, F., Werler, P.J., Bousset, K., Furuya, K., Diffley, J.F., Carr, A.M., and Elledge, S.J. (2001a). Mrc1 transduces signals of DNA replication stress to activate Rad53. Nat. Cell Biol. *3*, 958–965.

Alcasabas, A.A., Osborn, A.J., Bachant, J., Hu, F., Werler, P.J., Bousset, K., Furuya, K., Diffley, J.F., Carr, A.M., and Elledge, S.J. (2001b). Mrc1 transduces signals of DNA replication stress to activate Rad53. Nat. Cell Biol. *3*, 958–965.

Allen, J.B., Zhou, Z., Siede, W., Friedberg, E.C., and Elledge, S.J. (1994). The SAD1/RAD53 protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. Genes Dev. *8*, 2401–2415.

Amon, A., Irniger, S., and Nasmyth, K. (1994). Closing the cell cycle circle in yeast: G2 cyclin proteolysis initiated at mitosis persists until the activation of G1 cyclins in the next cycle. Cell *77*, 1037–1050.

Arias, E.E., and Walter, J.C. (2007). Strength in numbers: preventing rereplication via multiple mechanisms in eukaryotic cells. Genes Dev. *21*, 497–518.

Arnaudeau, C., Lundin, C., and Helleday, T. (2001). DNA double-strand breaks associated with replication forks are predominantly repaired by homologous recombination involving an exchange mechanism in mammalian cells. J. Mol. Biol. *307*, 1235–1245.

Aylon, Y., Liefshitz, B., and Kupiec, M. (2004a). The CDK regulates repair of double-strand breaks by homologous recombination during the cell cycle. EMBO J. *23*, 4868–4875.

Aylon, Y., Liefshitz, B., and Kupiec, M. (2004b). The CDK regulates repair of double-strand breaks by homologous recombination during the cell cycle. EMBO J. *23*, 4868–4875.

Bandyopadhyay, S., Mehta, M., Kuo, D., Sung, M.-K., Chuang, R., Jaehnig, E.J., Bodenmiller, B., Licon, K., Copeland, W., Shales, M., et al. (2010). Rewiring of genetic networks in response to DNA damage. Science *330*, 1385–1389.

Beckman, K.B., and Ames, B.N. (1997). Oxidative decay of DNA. J. Biol. Chem. *272*, 19633–19636.

Berggren, M., Gallegos, A., Gasdaska, J.R., Gasdaska, P.Y., Warneke, J., and Powis, G. (1996). Thioredoxin and thioredoxin reductase gene expression in human tumors and cell lines, and the effects of serum stimulation and hypoxia. Anticancer Res. *16*, 3459–3466.

Bianchi, V. (1986). Effects of hydroxyurea on deoxynucleotide pools of mouse fibroblasts. Prog. Clin. Biol. Res. *209A*, 533–539.

Bjergbaek, L., Cobb, J.A., Tsai-Pflugfelder, M., and Gasser, S.M. (2005). Mechanistically distinct roles for Sgs1p in checkpoint activation and replication fork maintenance. EMBO J. *24*, 405–417.

Boiteux, S., Gellon, L., and Guibourt, N. (2002). Repair of 8-oxoguanine in Saccharomyces cerevisiae: interplay of DNA repair and replication mechanisms. Free Radic. Biol. Med. *32*, 1244–1253.

Bonetti, D., Clerici, M., Manfrini, N., Lucchini, G., and Longhese, M.P. (2010). The MRX complex plays multiple functions in resection of Yku- and Rif2-protected DNA ends. PLoS ONE *5*, e14142.

Boos, D., Frigola, J., and Diffley, J.F.X. (2012). Activation of the replicative DNA helicase: breaking up is hard to do. Curr. Opin. Cell Biol. *24*, 423–430.

Botchan, M., and Berger, J. (2010). DNA replication: making two forks from one prereplication complex. Mol. Cell *40*, 860–861.

Branzei, D., and Foiani, M. (2007). Interplay of replication checkpoints and repair proteins at stalled replication forks. DNA Repair (Amst.) *6*, 994–1003.

Branzei, D., and Foiani, M. (2008). Regulation of DNA repair throughout the cell cycle. Nat. Rev. Mol. Cell Biol. *9*, 297–308.

Branzei, D., and Foiani, M. (2010). Maintaining genome stability at the replication fork. Nat. Rev. Mol. Cell Biol. *11*, 208–219.

Branzei, D., Sollier, J., Liberi, G., Zhao, X., Maeda, D., Seki, M., Enomoto, T., Ohta, K., and Foiani, M. (2006). Ubc9- and mms21-mediated sumoylation counteracts recombinogenic events at damaged replication forks. Cell *127*, 509–522.

Branzei, D., Vanoli, F., and Foiani, M. (2008). SUMOylation regulates Rad18-mediated template switch. Nature *456*, 915–920.

Byun, T.S., Pacek, M., Yee, M., Walter, J.C., and Cimprich, K.A. (2005). Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. Genes Dev. *19*, 1040–1052.

Bzymek, M., Thayer, N.H., Oh, S.D., Kleckner, N., and Hunter, N. (2010a). Double Holliday junctions are intermediates of DNA break repair. Nature *464*, 937–941.

Bzymek, M., Thayer, N.H., Oh, S.D., Kleckner, N., and Hunter, N. (2010b). Double Holliday junctions are intermediates of DNA break repair. Nature *464*, 937–941.

Cadet, J., Berger, M., Douki, T., and Ravanat, J.L. (1997). Oxidative damage to DNA: formation, measurement, and biological significance. Rev. Physiol. Biochem. Pharmacol. *131*, 1–87.

Camier, S., Ma, E., Leroy, C., Pruvost, A., Toledano, M., and Marsolier-Kergoat, M.-C. (2007). Visualization of ribonucleotide reductase catalytic oxidation establishes thioredoxins as its major reductants in yeast. Free Radic. Biol. Med. *42*, 1008–1016.

Carmel-Harel, O., Stearman, R., Gasch, A.P., Botstein, D., Brown, P.O., and Storz, G. (2001). Role of thioredoxin reductase in the Yap1p-dependent response to oxidative stress in Saccharomyces cerevisiae. Mol. Microbiol. *39*, 595–605.

Caspari, T., Murray, J.M., and Carr, A.M. (2002). Cdc2-cyclin B kinase activity links Crb2 and Rqh1-topoisomerase III. Genes Dev. *16*, 1195–1208.

Casper, A.M., Nghiem, P., Arlt, M.F., and Glover, T.W. (2002). ATR regulates fragile site stability. Cell *111*, 779–789.

Cha, R.S., and Kleckner, N. (2002). ATR homolog Mec1 promotes fork progression, thus averting breaks in replication slow zones. Science *297*, 602–606.

Chabes, A., Domkin, V., and Thelander, L. (1999). Yeast Sml1, a protein inhibitor of ribonucleotide reductase. J. Biol. Chem. *274*, 36679–36683.

Chabes, A., Georgieva, B., Domkin, V., Zhao, X., Rothstein, R., and Thelander, L. (2003). Survival of DNA damage in yeast directly depends on increased dNTP levels allowed by relaxed feedback inhibition of ribonucleotide reductase. Cell *112*, 391–401.

Chae, H.Z., Chung, S.J., and Rhee, S.G. (1994). Thioredoxin-dependent peroxide reductase from yeast. J. Biol. Chem. *269*, 27670–27678.

Charizanis, C., Juhnke, H., Krems, B., and Entian, K.D. (1999). The oxidative stress response mediated via Pos9/Skn7 is negatively regulated by the Ras/PKA pathway in Saccharomyces cerevisiae. Mol. Gen. Genet. *261*, 740–752.

Chen, H., Lisby, M., and Symington, L.S. (2013). RPA coordinates DNA end resection and prevents formation of DNA hairpins. Mol. Cell *50*, 589–600.

Chen, X., Niu, H., Chung, W.-H., Zhu, Z., Papusha, A., Shim, E.Y., Lee, S.E., Sung, P., and Ira, G. (2011). Cell cycle regulation of DNA double-strand break end resection by Cdk1-dependent Dna2 phosphorylation. Nat. Struct. Mol. Biol. *18*, 1015–1019.

Chilkova, O., Stenlund, P., Isoz, I., Stith, C.M., Grabowski, P., Lundström, E.-B., Burgers, P.M., and Johansson, E. (2007). The eukaryotic leading and lagging strand DNA polymerases are loaded onto primer-ends via separate mechanisms but have comparable processivity in the presence of PCNA. Nucleic Acids Res. *35*, 6588–6597.

Clerici, M., Mantiero, D., Guerini, I., Lucchini, G., and Longhese, M.P. (2008). The Yku70-Yku80 complex contributes to regulate double-strand break processing and checkpoint activation during the cell cycle. EMBO Rep. *9*, 810–818.

Coleman, S.T., Epping, E.A., Steggerda, S.M., and Moye-Rowley, W.S. (1999). Yap1p activates gene transcription in an oxidant-specific fashion. Mol. Cell. Biol. *19*, 8302–8313.

D'Amours, D., and Jackson, S.P. (2001). The yeast Xrs2 complex functions in S phase checkpoint regulation. Genes Dev. *15*, 2238–2249.

D'Amours, D., and Jackson, S.P. (2002). The Mre11 complex: at the crossroads of dna repair and checkpoint signalling. Nat. Rev. Mol. Cell Biol. *3*, 317–327.

Daley, J.M., Palmbos, P.L., Wu, D., and Wilson, T.E. (2005). Nonhomologous end joining in yeast. Annu. Rev. Genet. *39*, 431–451.

Davies, A.A., Huttner, D., Daigaku, Y., Chen, S., and Ulrich, H.D. (2008). Activation of ubiquitin-dependent DNA damage bypass is mediated by replication protein a. Mol. Cell *29*, 625–636.

Demple, B., and Harrison, L. (1994). Repair of oxidative damage to DNA: enzymology and biology. Annu. Rev. Biochem. *63*, 915–948.

Desany, B.A., Alcasabas, A.A., Bachant, J.B., and Elledge, S.J. (1998). Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway. Genes Dev. *12*, 2956–2970.

Desdouets, C., Santocanale, C., Drury, L.S., Perkins, G., Foiani, M., Plevani, P., and Diffley, J.F. (1998). Evidence for a Cdc6p-independent mitotic resetting event involving DNA polymerase alpha. EMBO J. *17*, 4139–4146.

Dizdaroglu, M. (1991). Chemical determination of free radical-induced damage to DNA. Free Radic. Biol. Med. *10*, 225–242.

Draculic, T., Dawes, I.W., and Grant, C.M. (2000). A single glutaredoxin or thioredoxin gene is essential for viability in the yeast Saccharomyces cerevisiae. Mol. Microbiol. *36*, 1167–1174.

Durkin, S.G., and Glover, T.W. (2007). Chromosome fragile sites. Annu. Rev. Genet. *41*, 169–192.

Errico, A., and Costanzo, V. (2012). Mechanisms of replication fork protection: a safeguard for genome stability. Crit. Rev. Biochem. Mol. Biol. *47*, 222–235.

Evans, J., Maccabee, M., Hatahet, Z., Courcelle, J., Bockrath, R., Ide, H., and Wallace, S. (1993). Thymine ring saturation and fragmentation products: lesion bypass, misinsertion and implications for mutagenesis. Mutat. Res. *299*, 147–156.

Fishman-Lobell, J., Rudin, N., and Haber, J.E. (1992). Two alternative pathways of double-strand break repair that are kinetically separable and independently modulated. Mol. Cell. Biol. *12*, 1292–1303.

Foiani, M., Ferrari, M., Liberi, G., Lopes, M., Lucca, C., Marini, F., Pellicioli, A., Muzi Falconi, M., and Plevani, P. (1998). S-phase DNA damage checkpoint in budding yeast. Biol. Chem. *379*, 1019–1023.

Fraga, C.G., Shigenaga, M.K., Park, J.W., Degan, P., and Ames, B.N. (1990). Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. Proc. Natl. Acad. Sci. U.S.A. *87*, 4533–4537.

Frank-Vaillant, M., and Marcand, S. (2002). Transient stability of DNA ends allows nonhomologous end joining to precede homologous recombination. Mol. Cell *10*, 1189–1199.

Friedel, A.M., Pike, B.L., and Gasser, S.M. (2009). ATR/Mec1: coordinating fork stability and repair. Curr. Opin. Cell Biol. *21*, 237–244.

Gajewski, E., Rao, G., Nackerdien, Z., and Dizdaroglu, M. (1990). Modification of DNA bases in mammalian chromatin by radiation-generated free radicals. Biochemistry *29*, 7876–7882.

Gallo-Fernández, M., Saugar, I., Ortiz-Bazán, M.Á., Vázquez, M.V., and Tercero, J.A. (2012). Cell cycle-dependent regulation of the nuclease activity of Mus81-Eme1/Mms4. Nucleic Acids Res. *40*, 8325–8335.

Gan, Z.R. (1991). Yeast thioredoxin genes. J. Biol. Chem. *266*, 1692–1696.

Gan, Z.R. (1992). Cloning and sequencing of a gene encoding yeast thioltransferase. Biochem. Biophys. Res. Commun. *187*, 949–955.

Garrido, E.O., and Grant, C.M. (2002). Role of thioredoxins in the response of Saccharomyces cerevisiae to oxidative stress induced by hydroperoxides. Mol. Microbiol. *43*, 993–1003.

Gravel, S., Chapman, J.R., Magill, C., and Jackson, S.P. (2008). DNA helicases Sgs1 and BLM promote DNA double-strand break resection. Genes Dev. *22*, 2767–2772.

Grollman, A.P., and Moriya, M. (1993). Mutagenesis by 8-oxoguanine: an enemy within. Trends Genet. *9*, 246–249.

Halliwell, B. (2006). Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol. *141*, 312–322.

Harrison, J.C., and Haber, J.E. (2006). Surviving the breakup: the DNA damage checkpoint. Annu. Rev. Genet. *40*, 209–235.

Harshman, K.D., Moye-Rowley, W.S., and Parker, C.S. (1988). Transcriptional activation by the SV40 AP-1 recognition element in yeast is mediated by a factor similar to AP-1 that is distinct from GCN4. Cell *53*, 321–330.

Helbock, H.J., Beckman, K.B., Shigenaga, M.K., Walter, P.B., Woodall, A.A., Yeo, H.C., and Ames, B.N. (1998). DNA oxidation matters: the HPLC-electrochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guanine. Proc. Natl. Acad. Sci. U.S.A. *95*, 288–293.

Heller, R.C., and Marians, K.J. (2006). Replication fork reactivation downstream of a blocked nascent leading strand. Nature *439*, 557–562.

Heyer, W.-D. (2007). Biochemistry of eukaryotic homologous recombination. Top Curr Genet *17*, 95–133.

Heyer, W.-D., Ehmsen, K.T., and Liu, J. (2010). Regulation of homologous recombination in eukaryotes. Annu. Rev. Genet. *44*, 113–139.

Hiltunen, J.K., Mursula, A.M., Rottensteiner, H., Wierenga, R.K., Kastaniotis, A.J., and Gurvitz, A. (2003). The biochemistry of peroxisomal beta-oxidation in the yeast Saccharomyces cerevisiae. FEMS Microbiol. Rev. *27*, 35–64.

Holloman, W.K. (2011). Unraveling the mechanism of BRCA2 in homologous recombination. Nat. Struct. Mol. Biol. *18*, 748–754.

Holthausen, J.T., Wyman, C., and Kanaar, R. (2010). Regulation of DNA strand exchange in homologous recombination. DNA Repair (Amst.) *9*, 1264–1272.

Huertas, P., and Jackson, S.P. (2009). Human CtIP mediates cell cycle control of DNA end resection and double strand break repair. J. Biol. Chem. *284*, 9558–9565.

Huertas, P., Cortés-Ledesma, F., Sartori, A.A., Aguilera, A., and Jackson, S.P. (2008a). CDK targets Sae2 to control DNA-end resection and homologous recombination. Nature *455*, 689–692.

Huertas, P., Cortés-Ledesma, F., Sartori, A.A., Aguilera, A., and Jackson, S.P. (2008b). CDK targets Sae2 to control DNA-end resection and homologous recombination. Nature *455*, 689–692.

Hunter, N., and Kleckner, N. (2001). The single-end invasion: an asymmetric intermediate at the double-strand break to double-holliday junction transition of meiotic recombination. Cell *106*, 59–70.

Hussain, M., and Lenard, J. (1991). Characterization of PDR4, a Saccharomyces cerevisiae gene that confers pleiotropic drug resistance in high-copy number: identity with YAP1, encoding a transcriptional activator [corrected]. Gene *101*, 149–152.

Ira, G., Malkova, A., Liberi, G., Foiani, M., and Haber, J.E. (2003). Srs2 and Sgs1-Top3 suppress crossovers during double-strand break repair in yeast. Cell *115*, 401–411.

Ira, G., Pellicioli, A., Balijja, A., Wang, X., Fiorani, S., Carotenuto, W., Liberi, G., Bressan, D., Wan, L., Hollingsworth, N.M., et al. (2004a). DNA end resection, homologous recombination and DNA damage checkpoint activation require CDK1. Nature *431*, 1011–1017.

Ira, G., Pellicioli, A., Balijja, A., Wang, X., Fiorani, S., Carotenuto, W., Liberi, G., Bressan, D., Wan, L., Hollingsworth, N.M., et al. (2004b). DNA end resection, homologous recombination and DNA damage checkpoint activation require CDK1. Nature *431*, 1011–1017.

Iraqui, I., Kienda, G., Soeur, J., Faye, G., Baldacci, G., Kolodner, R.D., and Huang, M.-E. (2009). Peroxiredoxin Tsa1 is the key peroxidase suppressing genome instability and protecting against cell death in Saccharomyces cerevisiae. PLoS Genet. *5*, e1000524.

Ivanov, E.L., Sugawara, N., Fishman-Lobell, J., and Haber, J.E. (1996). Genetic requirements for the single-strand annealing pathway of double-strand break repair in Saccharomyces cerevisiae. Genetics *142*, 693–704.

Izawa, S., Maeda, K., Sugiyama, K., Mano, J., Inoue, Y., and Kimura, A. (1999). Thioredoxin deficiency causes the constitutive activation of Yap1, an AP-1-like transcription factor in Saccharomyces cerevisiae. J. Biol. Chem. *274*, 28459–28465.

Jain, S., Sugawara, N., Lydeard, J., Vaze, M., Tanguy Le Gac, N., and Haber, J.E. (2009). A recombination execution checkpoint regulates the choice of homologous recombination pathway during DNA double-strand break repair. Genes Dev. *23*, 291–303.

Jessop, L., Rockmill, B., Roeder, G.S., and Lichten, M. (2006). Meiotic chromosome synapsis-promoting proteins antagonize the anti-crossover activity of sgs1. PLoS Genet. *2*, e155.

Kai, M., Furuya, K., Paderi, F., Carr, A.M., and Wang, T.S.F. (2007). Rad3-dependent phosphorylation of the checkpoint clamp regulates repair-pathway choice. Nat. Cell Biol. *9*, 691–697.

Kasai, H., and Nishimura, S. (1984). DNA damage induced by asbestos in the presence of hydrogen peroxide. Gann *75*, 841–844.

Koc, A., Mathews, C.K., Wheeler, L.J., Gross, M.K., and Merrill, G.F. (2006). Thioredoxin is required for deoxyribonucleotide pool maintenance during S phase. J. Biol. Chem. *281*, 15058–15063.

Kolodner, R.D., Putnam, C.D., and Myung, K. (2002). Maintenance of genome stability in Saccharomyces cerevisiae. Science *297*, 552–557.

Krejci, L., Van Komen, S., Li, Y., Villemain, J., Reddy, M.S., Klein, H., Ellenberger, T., and Sung, P. (2003). DNA helicase Srs2 disrupts the Rad51 presynaptic filament. Nature *423*, 305–309.

Krems, B., Charizanis, C., and Entian, K.D. (1996). The response regulator-like protein Pos9/Skn7 of Saccharomyces cerevisiae is involved in oxidative stress resistance. Curr. Genet. *29*, 327–334.

Krogh, B.O., and Symington, L.S. (2004a). Recombination proteins in yeast. Annu. Rev. Genet. *38*, 233–271.

Krogh, B.O., and Symington, L.S. (2004b). Recombination proteins in yeast. Annu. Rev. Genet. *38*, 233–271.

Kuge, S., and Jones, N. (1994). YAP1 dependent activation of TRX2 is essential for the response of Saccharomyces cerevisiae to oxidative stress by hydroperoxides. EMBO J. *13*, 655–664.

Kuge, S., Jones, N., and Nomoto, A. (1997). Regulation of yAP-1 nuclear localization in response to oxidative stress. EMBO J. *16*, 1710–1720.

Kuge, S., Toda, T., Iizuka, N., and Nomoto, A. (1998). Crm1 (XpoI) dependent nuclear export of the budding yeast transcription factor yAP-1 is sensitive to oxidative stress. Genes Cells *3*, 521–532.

Kuraoka, I., Bender, C., Romieu, A., Cadet, J., Wood, R.D., and Lindahl, T. (2000). Removal of oxygen free-radical-induced 5',8-purine cyclodeoxynucleosides from DNA by the nucleotide excision-repair pathway in human cells. Proc. Natl. Acad. Sci. U.S.A. *97*, 3832–3837.

Labib, K. (2010). How do Cdc7 and cyclin-dependent kinases trigger the initiation of chromosome replication in eukaryotic cells? Genes Dev. *24*, 1208–1219.

Lambert, S., Froget, B., and Carr, A.M. (2007). Arrested replication fork processing: interplay between checkpoints and recombination. DNA Repair (Amst.) *6*, 1042–1061.

Larrea, A.A., Lujan, S.A., Nick McElhinny, S.A., Mieczkowski, P.A., Resnick, M.A., Gordenin, D.A., and Kunkel, T.A. (2010). Genome-wide model for the normal eukaryotic DNA replication fork. Proc. Natl. Acad. Sci. U.S.A. *107*, 17674–17679.

LAURENT, T.C., MOORE, E.C., and REICHARD, P. (1964). ENZYMATIC SYNTHESIS OF DEOXYRIBONUCLEOTIDES. IV. ISOLATION AND CHARACTERIZATION OF THIOREDOXIN, THE HYDROGEN DONOR FROM ESCHERICHIA COLI B. J. Biol. Chem. *239*, 3436–3444.

Laureti, L., Selva, M., Dairou, J., and Matic, I. (2013). Reduction of dNTP levels enhances DNA replication fidelity in vivo. DNA Repair (Amst.) *12*, 300–305.

Lazzaro, F., Sapountzi, V., Granata, M., Pellicioli, A., Vaze, M., Haber, J.E., Plevani, P., Lydall, D., and Muzi-Falconi, M. (2008). Histone methyltransferase Dot1 and Rad9 inhibit single-stranded DNA accumulation at DSBs and uncapped telomeres. EMBO J. *27*, 1502–1512.

Lee, J., Godon, C., Lagniel, G., Spector, D., Garin, J., Labarre, J., and Toledano, M.B. (1999). Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. J. Biol. Chem. *274*, 16040–16046.

Lee, S.E., Moore, J.K., Holmes, A., Umezu, K., Kolodner, R.D., and Haber, J.E. (1998). Saccharomyces Ku70, mre11/rad50 and RPA proteins regulate adaptation to G2/M arrest after DNA damage. Cell *94*, 399–409.

Lemoine, F.J., Degtyareva, N.P., Lobachev, K., and Petes, T.D. (2005). Chromosomal translocations in yeast induced by low levels of DNA polymerase a model for chromosome fragile sites. Cell *120*, 587–598.

Lennon, B.W., Williams, C.H., Jr, and Ludwig, M.L. (2000). Twists in catalysis: alternating conformations of Escherichia coli thioredoxin reductase. Science *289*, 1190–1194.

Leppert, G., McDevitt, R., Falco, S.C., Van Dyk, T.K., Ficke, M.B., and Golin, J. (1990). Cloning by gene amplification of two loci conferring multiple drug resistance in Saccharomyces. Genetics *125*, 13–20.

Leroy, C., Mann, C., and Marsolier, M.C. (2001). Silent repair accounts for cell cycle specificity in the signaling of oxidative DNA lesions. EMBO J. *20*, 2896–2906.

Li, X., and Heyer, W.-D. (2008). Homologous recombination in DNA repair and DNA damage tolerance. Cell Res. *18*, 99–113.

Liao, S., Toczylowski, T., and Yan, H. (2008). Identification of the Xenopus DNA2 protein as a major nuclease for the 5'->3' strand-specific processing of DNA ends. Nucleic Acids Res. *36*, 6091–6100.

Lincoln, D.T., Ali Emadi, E.M., Tonissen, K.F., and Clarke, F.M. (2003). The thioredoxin-thioredoxin reductase system: over-expression in human cancer. Anticancer Res. *23*, 2425–2433.

Lindahl, T. (1993). Instability and decay of the primary structure of DNA. Nature *362*, 709–715.

Van der Linden, E., Sanchez, H., Kinoshita, E., Kanaar, R., and Wyman, C. (2009). RAD50 and NBS1 form a stable complex functional in DNA binding and tethering. Nucleic Acids Res. *37*, 1580–1588.

Lisby, M., Rothstein, R., and Mortensen, U.H. (2001). Rad52 forms DNA repair and recombination centers during S phase. Proc. Natl. Acad. Sci. U.S.A. *98*, 8276–8282.

Lisby, M., Antúnez de Mayolo, A., Mortensen, U.H., and Rothstein, R. (2003). Cell cycle-regulated centers of DNA double-strand break repair. Cell Cycle *2*, 479–483.

Lo, Y.-C., Paffett, K.S., Amit, O., Clikeman, J.A., Sterk, R., Brenneman, M.A., and Nickoloff, J.A. (2006). Sgs1 regulates gene conversion tract lengths and crossovers independently of its helicase activity. Mol. Cell. Biol. *26*, 4086–4094.

Longhese, M.P., Bonetti, D., Manfrini, N., and Clerici, M. (2010). Mechanisms and regulation of DNA end resection. EMBO J. *29*, 2864–2874.

Lopes, M., Cotta-Ramusino, C., Pellicioli, A., Liberi, G., Plevani, P., Muzi-Falconi, M., Newlon, C.S., and Foiani, M. (2001). The DNA replication checkpoint response stabilizes stalled replication forks. Nature *412*, 557–561.

Lopes, M., Foiani, M., and Sogo, J.M. (2006). Multiple mechanisms control chromosome integrity after replication fork uncoupling and restart at irreparable UV lesions. Mol. Cell *21*, 15–27.

Lundin, C., Erixon, K., Arnaudeau, C., Schultz, N., Jenssen, D., Meuth, M., and Helleday, T. (2002). Different roles for nonhomologous end joining and homologous recombination following replication arrest in mammalian cells. Mol. Cell. Biol. *22*, 5869–5878.

Lupardus, P.J., Byun, T., Yee, M.-C., Hekmat-Nejad, M., and Cimprich, K.A. (2002). A requirement for replication in activation of the ATR-dependent DNA damage checkpoint. Genes Dev. *16*, 2327–2332.

Macneill, S. (2012). Composition and dynamics of the eukaryotic replisome: a brief overview. Subcell. Biochem. *62*, 1–17.

Malumbres, M., and Barbacid, M. (2005). Mammalian cyclin-dependent kinases. Trends Biochem. Sci. *30*, 630–641.

Martini, E., Diaz, R.L., Hunter, N., and Keeney, S. (2006). Crossover homeostasis in yeast meiosis. Cell *126*, 285–295.

Matos, J., Blanco, M.G., Maslen, S., Skehel, J.M., and West, S.C. (2011). Regulatory control of the resolution of DNA recombination intermediates during meiosis and mitosis. Cell *147*, 158–172.

Meyer, Y., Buchanan, B.B., Vignols, F., and Reichheld, J.-P. (2009a). Thioredoxins and glutaredoxins: unifying elements in redox biology. Annu. Rev. Genet. *43*, 335–367.

Meyer, Y., Buchanan, B.B., Vignols, F., and Reichheld, J.-P. (2009b). Thioredoxins and glutaredoxins: unifying elements in redox biology. Annu. Rev. Genet. *43*, 335–367.

Mimitou, E.P., and Symington, L.S. (2008). Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing. Nature *455*, 770–774.

Mimitou, E.P., and Symington, L.S. (2009). DNA end resection: many nucleases make light work. DNA Repair (Amst.) *8*, 983–995.

Mimitou, E.P., and Symington, L.S. (2011). DNA end resection--unraveling the tail. DNA Repair (Amst.) *10*, 344–348.

Mirkin, S.M. (2006). DNA structures, repeat expansions and human hereditary disorders. Curr. Opin. Struct. Biol. *16*, 351–358.

Mirkin, E.V., and Mirkin, S.M. (2007). Replication fork stalling at natural impediments. Microbiol. Mol. Biol. Rev. *71*, 13–35.

Miyabe, I., Kunkel, T.A., and Carr, A.M. (2011). The major roles of DNA polymerases epsilon and delta at the eukaryotic replication fork are evolutionarily conserved. PLoS Genet. *7*, e1002407.

Morano, K.A., Grant, C.M., and Moye-Rowley, W.S. (2012). The response to heat shock and oxidative stress in Saccharomyces cerevisiae. Genetics *190*, 1157–1195.

Morgan, B.A., Banks, G.R., Toone, W.M., Raitt, D., Kuge, S., and Johnston, L.H. (1997). The Skn7 response regulator controls gene expression in the oxidative stress response of the budding yeast Saccharomyces cerevisiae. EMBO J. *16*, 1035–1044.

Morris, J.R., Boutell, C., Keppler, M., Densham, R., Weekes, D., Alamshah, A., Butler, L., Galanty, Y., Pangon, L., Kiuchi, T., et al. (2009). The SUMO modification pathway is involved in the BRCA1 response to genotoxic stress. Nature *462*, 886–890.

Mouaheb, N., Thomas, D., Verdoucq, L., Monfort, P., and Meyer, Y. (1998). In vivo functional discrimination between plant thioredoxins by heterologous expression in the yeast Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U.S.A. *95*, 3312–3317.

Moye-Rowley, W.S., Harshman, K.D., and Parker, C.S. (1989). Yeast YAP1 encodes a novel form of the jun family of transcriptional activator proteins. Genes Dev. *3*, 283–292.

Muller, E.G. (1991). Thioredoxin deficiency in yeast prolongs S phase and shortens the G1 interval of the cell cycle. J. Biol. Chem. *266*, 9194–9202.

Muller, E.G. (1992). Thioredoxin genes in Saccharomyces cerevisiae: map positions of TRX1 and TRX2. Yeast *8*, 117–120.

Muller, E.G. (1995). A redox-dependent function of thioredoxin is necessary to sustain a rapid rate of DNA synthesis in yeast. Arch. Biochem. Biophys. *318*, 356–361.

Murphy, M.P. (2009). How mitochondria produce reactive oxygen species. Biochem. J. *417*, 1–13.

Myung, K., Datta, A., and Kolodner, R.D. (2001a). Suppression of spontaneous chromosomal rearrangements by S phase checkpoint functions in Saccharomyces cerevisiae. Cell *104*, 397–408.

Myung, K., Datta, A., and Kolodner, R.D. (2001b). Suppression of spontaneous chromosomal rearrangements by S phase checkpoint functions in Saccharomyces cerevisiae. Cell *104*, 397–408.

Neale, M.J., Pan, J., and Keeney, S. (2005). Endonucleolytic processing of covalent protein-linked DNA double-strand breaks. Nature *436*, 1053–1057.

Nedelcheva, M.N., Roguev, A., Dolapchiev, L.B., Shevchenko, A., Taskov, H.B., Shevchenko, A., Stewart, A.F., and Stoynov, S.S. (2005). Uncoupling of unwinding from DNA synthesis implies regulation of MCM helicase by Tof1/Mrc1/Csm3 checkpoint complex. J. Mol. Biol. *347*, 509–521.

Nick McElhinny, S.A., Gordenin, D.A., Stith, C.M., Burgers, P.M.J., and Kunkel, T.A. (2008). Division of labor at the eukaryotic replication fork. Mol. Cell *30*, 137–144.

Núñez-Ramírez, R., Klinge, S., Sauguet, L., Melero, R., Recuero-Checa, M.A., Kilkenny, M., Perera, R.L., García-Alvarez, B., Hall, R.J., Nogales, E., et al. (2011). Flexible tethering of primase and DNA Pol α in the eukaryotic primosome. Nucleic Acids Res. *39*, 8187–8199.

Ohtake, Y., and Yabuuchi, S. (1991). Molecular cloning of the gamma-glutamylcysteine synthetase gene of Saccharomyces cerevisiae. Yeast *7*, 953–961.

Okazaki, S., Tachibana, T., Naganuma, A., Mano, N., and Kuge, S. (2007). Multistep disulfide bond formation in Yap1 is required for sensing and transduction of H2O2 stress signal. Mol. Cell *27*, 675–688.

Oliveira, M.A., Discola, K.F., Alves, S.V., Medrano, F.J., Guimarães, B.G., and Netto, L.E.S. (2010). Insights into the specificity of thioredoxin reductase-thioredoxin interactions. A structural and functional investigation of the yeast thioredoxin system. Biochemistry *49*, 3317–3326.

Orr, H.T., and Zoghbi, H.Y. (2007). Trinucleotide repeat disorders. Annu. Rev. Neurosci. *30*, 575–621.

Pâques, F., and Haber, J.E. (1999a). Multiple pathways of recombination induced by double-strand breaks in Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev. *63*, 349–404.

Pâques, F., and Haber, J.E. (1999b). Multiple pathways of recombination induced by double-strand breaks in Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev. *63*, 349–404.

Paulovich, A.G., and Hartwell, L.H. (1995). A checkpoint regulates the rate of progression through S phase in S. cerevisiae in response to DNA damage. Cell *82*, 841–847.

Paulsen, R.D., and Cimprich, K.A. (2007). The ATR pathway: fine-tuning the fork. DNA Repair (Amst.) *6*, 953–966.

Pedrajas, J.R., Kosmidou, E., Miranda-Vizuete, A., Gustafsson, J.A., Wright, A.P., and Spyrou, G. (1999). Identification and functional characterization of a novel mitochondrial thioredoxin system in Saccharomyces cerevisiae. J. Biol. Chem. *274*, 6366–6373.

Petermann, E., and Helleday, T. (2010). Pathways of mammalian replication fork restart. Nat. Rev. Mol. Cell Biol. *11*, 683–687.

Petermann, E., Orta, M.L., Issaeva, N., Schultz, N., and Helleday, T. (2010). Hydroxyurea-stalled replication forks become progressively inactivated and require two different RAD51-mediated pathways for restart and repair. Mol. Cell *37*, 492–502.

Pfander, B., Moldovan, G.-L., Sacher, M., Hoege, C., and Jentsch, S. (2005). SUMO-modified PCNA recruits Srs2 to prevent recombination during S phase. Nature *436*, 428–433.

Prakash, R., Satory, D., Dray, E., Papusha, A., Scheller, J., Kramer, W., Krejci, L., Klein, H., Haber, J.E., Sung, P., et al. (2009). Yeast Mph1 helicase dissociates Rad51-made D-loops: implications for crossover control in mitotic recombination. Genes Dev. *23*, 67–79.

Prakash, S., Johnson, R.E., and Prakash, L. (2005). Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function. Annu. Rev. Biochem. *74*, 317–353.

Pursell, Z.F., Isoz, I., Lundström, E.-B., Johansson, E., and Kunkel, T.A. (2007). Yeast DNA polymerase epsilon participates in leading-strand DNA replication. Science *317*, 127–130.

Raitt, D.C., Johnson, A.L., Erkine, A.M., Makino, K., Morgan, B., Gross, D.S., and Johnston, L.H. (2000). The Skn7 response regulator of Saccharomyces cerevisiae interacts with Hsf1 in vivo and is required for the induction of heat shock genes by oxidative stress. Mol. Biol. Cell *11*, 2335–2347.

Reagan, M.S., Pittenger, C., Siede, W., and Friedberg, E.C. (1995). Characterization of a mutant strain of Saccharomyces cerevisiae with a deletion of the RAD27 gene, a structural homolog of the RAD2 nucleotide excision repair gene. J. Bacteriol. *177*, 364–371.

Reichard, P. (1988). Interactions between deoxyribonucleotide and DNA synthesis. Annu. Rev. Biochem. *57*, 349–374.

Remus, D., Beuron, F., Tolun, G., Griffith, J.D., Morris, E.P., and Diffley, J.F.X. (2009). Concerted loading of Mcm2-7 double hexamers around DNA during DNA replication origin licensing. Cell *139*, 719–730.

Robert, T., Dervins, D., Fabre, F., and Gangloff, S. (2006). Mrc1 and Srs2 are major actors in the regulation of spontaneous crossover. EMBO J. *25*, 2837–2846.

Rowley, A., Johnston, G.C., Butler, B., Werner-Washburne, M., and Singer, R.A. (1993). Heat shock-mediated cell cycle blockage and G1 cyclin expression in the yeast Saccharomyces cerevisiae. Mol. Cell. Biol. *13*, 1034–1041.

Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998). Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. EMBO J. *17*, 2596–2606.

San Filippo, J., Sung, P., and Klein, H. (2008a). Mechanism of eukaryotic homologous recombination. Annu. Rev. Biochem. *77*, 229–257.

San Filippo, J., Sung, P., and Klein, H. (2008b). Mechanism of eukaryotic homologous recombination. Annu. Rev. Biochem. *77*, 229–257.

Sanchez, Y., Desany, B.A., Jones, W.J., Liu, Q., Wang, B., and Elledge, S.J. (1996). Regulation of RAD53 by the ATM-like kinases MEC1 and TEL1 in yeast cell cycle checkpoint pathways. Science *271*, 357–360.

Santocanale, C., and Diffley, J.F. (1998). A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication. Nature *395*, 615–618.

Saponaro, M., Callahan, D., Zheng, X., Krejci, L., Haber, J.E., Klein, H.L., and Liberi, G. (2010). Cdk1 targets Srs2 to complete synthesis-dependent strand annealing and to promote recombinational repair. PLoS Genet. *6*, e1000858.

Sartori, A.A., Lukas, C., Coates, J., Mistrik, M., Fu, S., Bartek, J., Baer, R., Lukas, J., and Jackson, S.P. (2007). Human CtIP promotes DNA end resection. Nature *450*, 509–514.

Schiestl, R.H., Prakash, S., and Prakash, L. (1990). The SRS2 suppressor of rad6 mutations of Saccharomyces cerevisiae acts by channeling DNA lesions into the RAD52 DNA repair pathway. Genetics *124*, 817–831.

Schnell, N., Krems, B., and Entian, K.D. (1992). The PAR1 (YAP1/SNQ3) gene of Saccharomyces cerevisiae, a c-jun homologue, is involved in oxygen metabolism. Curr. Genet. *21*, 269–273.

Schwob, E., Böhm, T., Mendenhall, M.D., and Nasmyth, K. (1994). The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in S. cerevisiae. Cell *79*, 233–244.

Segurado, M., and Tercero, J.A. (2009a). The S-phase checkpoint: targeting the replication fork. Biol. Cell *101*, 617–627.

Segurado, M., and Tercero, J.A. (2009b). The S-phase checkpoint: targeting the replication fork. Biol. Cell *101*, 617–627.

Sharples, G.J., and Leach, D.R. (1995). Structural and functional similarities between the SbcCD proteins of Escherichia coli and the RAD50 and MRE11 (RAD32) recombination and repair proteins of yeast. Mol. Microbiol. *17*, 1215–1217.

Shcherbakova, P.V., Pavlov, Y.I., Chilkova, O., Rogozin, I.B., Johansson, E., and Kunkel, T.A. (2003). Unique error signature of the four-subunit yeast DNA polymerase epsilon. J. Biol. Chem. *278*, 43770–43780.

Shibata, A., Conrad, S., Birraux, J., Geuting, V., Barton, O., Ismail, A., Kakarougkas, A., Meek, K., Taucher-Scholz, G., Löbrich, M., et al. (2011). Factors determining DNA double-strand break repair pathway choice in G2 phase. EMBO J. *30*, 1079–1092.

Shim, E.Y., Chung, W.-H., Nicolette, M.L., Zhang, Y., Davis, M., Zhu, Z., Paull, T.T., Ira, G., and Lee, S.E. (2010a). Saccharomyces cerevisiae Mre11/Rad50/Xrs2 and Ku proteins regulate association of Exo1 and Dna2 with DNA breaks. EMBO J. *29*, 3370–3380.

Shim, E.Y., Chung, W.-H., Nicolette, M.L., Zhang, Y., Davis, M., Zhu, Z., Paull, T.T., Ira, G., and Lee, S.E. (2010b). Saccharomyces cerevisiae Mre11/Rad50/Xrs2 and Ku proteins regulate association of Exo1 and Dna2 with DNA breaks. EMBO J. *29*, 3370–3380.

Shirahige, K., Hori, Y., Shiraishi, K., Yamashita, M., Takahashi, K., Obuse, C., Tsurimoto, T., and Yoshikawa, H. (1998). Regulation of DNA-replication origins during cell-cycle progression. Nature *395*, 618–621.

Sogo, J.M., Lopes, M., and Foiani, M. (2002). Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. Science *297*, 599–602.

Stokes, M.P., Van Hatten, R., Lindsay, H.D., and Michael, W.M. (2002). DNA replication is required for the checkpoint response to damaged DNA in Xenopus egg extracts. J. Cell Biol. *158*, 863–872.

Stracker, T.H., and Petrini, J.H.J. (2011). The MRE11 complex: starting from the ends. Nat. Rev. Mol. Cell Biol. *12*, 90–103.

Sung, P. (1997). Function of yeast Rad52 protein as a mediator between replication protein A and the Rad51 recombinase. J. Biol. Chem. *272*, 28194–28197.

Surana, U., Amon, A., Dowzer, C., McGrew, J., Byers, B., and Nasmyth, K. (1993). Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. EMBO J. *12*, 1969–1978.

Svendsen, J.M., and Harper, J.W. (2010). GEN1/Yen1 and the SLX4 complex: Solutions to the problem of Holliday junction resolution. Genes Dev. *24*, 521–536.

Swanson, R.L., Morey, N.J., Doetsch, P.W., and Jinks-Robertson, S. (1999). Overlapping specificities of base excision repair, nucleotide excision repair, recombination, and translesion synthesis pathways for DNA base damage in Saccharomyces cerevisiae. Mol. Cell. Biol. *19*, 2929–2935.

Symington, L.S., and Gautier, J. (2011). Double-strand break end resection and repair pathway choice. Annu. Rev. Genet. *45*, 247–271.

Szyjka, S.J., Aparicio, J.G., Viggiani, C.J., Knott, S., Xu, W., Tavaré, S., and Aparicio, O.M. (2008). Rad53 regulates replication fork restart after DNA damage in Saccharomyces cerevisiae. Genes Dev. *22*, 1906–1920.

Tang, H.-M.V., Siu, K.-L., Wong, C.-M., and Jin, D.-Y. (2009). Loss of yeast peroxiredoxin Tsa1p induces genome instability through activation of the DNA damage checkpoint and elevation of dNTP levels. PLoS Genet. *5*, e1000697.

Tercero, J.A., and Diffley, J.F. (2001). Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint. Nature *412*, 553–557.

Tercero, J.A., Longhese, M.P., and Diffley, J.F.X. (2003a). A central role for DNA replication forks in checkpoint activation and response. Mol. Cell *11*, 1323–1336.

Tercero, J.A., Longhese, M.P., and Diffley, J.F.X. (2003b). A central role for DNA replication forks in checkpoint activation and response. Mol. Cell *11*, 1323–1336.

Tittel-Elmer, M., Alabert, C., Pasero, P., and Cobb, J.A. (2009). The MRX complex stabilizes the replisome independently of the S phase checkpoint during replication stress. EMBO J. *28*, 1142–1156.

Tittel-Elmer, M., Lengronne, A., Davidson, M.B., Bacal, J., François, P., Hohl, M., Petrini, J.H.J., Pasero, P., and Cobb, J.A. (2012). Cohesin association to replication sites depends on rad50 and promotes fork restart. Mol. Cell *48*, 98–108.

Toledano, M.B., Kumar, C., Le Moan, N., Spector, D., and Tacnet, F. (2007). The system biology of thiol redox system in Escherichia coli and yeast: Differential functions in oxidative stress, iron metabolism and DNA synthesis. FEBS Letters *581*, 3598–3607.

Tonissen, K.F., and Di Trapani, G. (2009). Thioredoxin system inhibitors as mediators of apoptosis for cancer therapy. Mol Nutr Food Res *53*, 87–103.

Tourrière, H., and Pasero, P. (2007a). Maintenance of fork integrity at damaged DNA and natural pause sites. DNA Repair (Amst.) *6*, 900–913.

Tourrière, H., and Pasero, P. (2007b). Maintenance of fork integrity at damaged DNA and natural pause sites. DNA Repair *6*, 900–913.

Trotter, E.W., and Grant, C.M. (2003). Non-reciprocal regulation of the redox state of the glutathione-glutaredoxin and thioredoxin systems. EMBO Rep. *4*, 184–188.

Trotter, E.W., and Grant, C.M. (2005). Overlapping roles of the cytoplasmic and mitochondrial redox regulatory systems in the yeast Saccharomyces cerevisiae. Eukaryotic Cell *4*, 392–400.

Tsaponina, O., Barsoum, E., Aström, S.U., and Chabes, A. (2011). Ixr1 is required for the expression of the ribonucleotide reductase Rnr1 and maintenance of dNTP pools. PLoS Genet. *7*, e1002061.

Tu, B.P., and Weissman, J.S. (2004). Oxidative protein folding in eukaryotes: mechanisms and consequences. J. Cell Biol. *164*, 341–346.

Ubersax, J.A., Woodbury, E.L., Quang, P.N., Paraz, M., Blethrow, J.D., Shah, K., Shokat, K.M., and Morgan, D.O. (2003). Targets of the cyclin-dependent kinase Cdk1. Nature *425*, 859–864.

Vaze, M.B., Pellicioli, A., Lee, S.E., Ira, G., Liberi, G., Arbel-Eden, A., Foiani, M., and Haber, J.E. (2002). Recovery from checkpoint-mediated arrest after repair of a double-strand break requires Srs2 helicase. Mol. Cell *10*, 373–385.

Veaute, X., Jeusset, J., Soustelle, C., Kowalczykowski, S.C., Le Cam, E., and Fabre, F. (2003). The Srs2 helicase prevents recombination by disrupting Rad51 nucleoprotein filaments. Nature *423*, 309–312.

Vignols, F., Bréhélin, C., Surdin-Kerjan, Y., Thomas, D., and Meyer, Y. (2005). A yeast two-hybrid knockout strain to explore thioredoxin-interacting proteins in vivo. Proc. Natl. Acad. Sci. U.S.A. *102*, 16729–16734.

Wakasugi, N., Tagaya, Y., Wakasugi, H., Mitsui, A., Maeda, M., Yodoi, J., and Tursz, T. (1990). Adult T-cell leukemia-derived factor/thioredoxin, produced by both human T-lymphotropic virus type I- and Epstein-Barr virus-transformed lymphocytes, acts as an autocrine growth factor and synergizes with interleukin 1 and interleukin 2. Proc. Natl. Acad. Sci. U.S.A. *87*, 8282–8286.

Welsh, S.J., Bellamy, W.T., Briehl, M.M., and Powis, G. (2002). The redox protein thioredoxin-1 (Trx-1) increases hypoxia-inducible factor 1alpha protein expression: Trx-1 overexpression results in increased vascular endothelial growth factor production and enhanced tumor angiogenesis. Cancer Res. *62*, 5089–5095.

West, S.C. (2003). Molecular views of recombination proteins and their control. Nat. Rev. Mol. Cell Biol. *4*, 435–445.

Williams, G.J., Lees-Miller, S.P., and Tainer, J.A. (2010). Mre11-Rad50-Nbs1 conformations and the control of sensing, signaling, and effector responses at DNA double-strand breaks. DNA Repair (Amst.) *9*, 1299–1306.

Wohlbold, L., and Fisher, R.P. (2009). Behind the wheel and under the hood: functions of cyclin-dependent kinases in response to DNA damage. DNA Repair (Amst.) *8*, 1018–1024.

Wu, L., and Hickson, I.D. (2003a). The Bloom's syndrome helicase suppresses crossing over during homologous recombination. Nature *426*, 870–874.

Wu, L., and Hickson, I.D. (2003b). The Bloom's syndrome helicase suppresses crossing over during homologous recombination. Nature *426*, 870–874.

Wu, Y., Kantake, N., Sugiyama, T., and Kowalczykowski, S.C. (2008). Rad51 protein controls Rad52-mediated DNA annealing. J. Biol. Chem. *283*, 14883–14892.

Yardimci, H., Loveland, A.B., Habuchi, S., van Oijen, A.M., and Walter, J.C. (2010). Uncoupling of sister replisomes during eukaryotic DNA replication. Mol. Cell *40*, 834–840.

Youds, J.L., and Boulton, S.J. (2011). The choice in meiosis - defining the factors that influence crossover or non-crossover formation. J. Cell. Sci. *124*, 501–513.

Yu, X., and Chen, J. (2004). DNA damage-induced cell cycle checkpoint control requires CtIP, a phosphorylation-dependent binding partner of BRCA1 C-terminal domains. Mol. Cell. Biol. *24*, 9478–9486.

Yu, X., Fu, S., Lai, M., Baer, R., and Chen, J. (2006). BRCA1 ubiquitinates its phosphorylation-dependent binding partner CtIP. Genes Dev. *20*, 1721–1726.

Yun, M.H., and Hiom, K. (2009). CtIP-BRCA1 modulates the choice of DNA double-strand-break repair pathway throughout the cell cycle. Nature *459*, 460–463.

Zhang, Z., Bao, R., Zhang, Y., Yu, J., Zhou, C.-Z., and Chen, Y. (2009). Crystal structure of Saccharomyces cerevisiae cytoplasmic thioredoxin reductase Trr1 reveals the structural basis for species-specific recognition of thioredoxin. Biochim. Biophys. Acta *1794*, 124–128.

Zhou, B.B., and Elledge, S.J. (2000a). The DNA damage response: putting checkpoints in perspective. Nature *408*, 433–439.

Zhou, B.B., and Elledge, S.J. (2000b). The DNA damage response: putting checkpoints in perspective. Nature *408*, 433–439.

Zhu, Z., Chung, W.-H., Shim, E.Y., Lee, S.E., and Ira, G. (2008). Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. Cell *134*, 981–994.

Nostro padre ci diceva sempre: "Vi auguro una vita onesta e libera, anche se sarà difficile"

Sophie Magdalena Scholl